

# **ISOFORM-SPECIFIC ROLES OF THE ADAPTOR PROTEIN SHCA IN CELL SIGNALING**

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## SUMMARY

ShcA is a *bona fide* adaptor protein without any enzymatic activity. Upon activation of receptor tyrosine kinases, ShcA associates with the receptor and becomes tyrosine phosphorylated. Phosphorylated ShcA recruits the Grb2/SOS complex to the membrane, where SOS stimulates the small GTPase Ras, resulting in the activation of the Ras/MAPK pathway. The fact that Grb2 binds directly to most of the receptor tyrosine kinases raises the question of how important is the role of Shc in mediating MAPK activation? Moreover, beside growth factor-induced MAPK activation, are there other pathways in which ShcA-mediated MAPK activation is relevant?

ShcA is expressed in three different isoforms: p46<sup>Shc</sup>, p52<sup>Shc</sup>, and p66<sup>Shc</sup>. These isoforms are all derived from a single gene and differ only in their N-terminal part. Although all isoforms are phosphorylated by receptor tyrosine kinases, and subsequently bind to Grb2, the p66<sup>Shc</sup> isoform does not seem to mediate MAPK activation. The individual contribution of p46<sup>Shc</sup> and p52<sup>Shc</sup> in mediating MAPK activation is also not clear. The fact that all isoforms are ubiquitously expressed, with some restrictions for p66<sup>Shc</sup>, complicates the experimental investigation of each isoform. Recently, p66<sup>Shc</sup> has been implicated in the regulation of apoptosis in response to oxidative stress.

Using siRNA, we established a system which allows isoform-specific knockdown of ShcA proteins in tissue culture. Further development of this technique enabled us to express a single isoform in the absence of endogenous protein. This so-called “knockdown-in” technique is applicable for most proteins which are expressed in multiple isoforms, and allows the investigation of specific mutations against a clear background without overexpression.

We used this technique to investigate the contribution of individual ShcA isoforms to EGF-induced MAPK activation in epithelial cells. Knockdown of all or single ShcA isoforms had no effect on EGF-induced Erk activation. Moreover, overexpression of p66<sup>Shc</sup> in non p66<sup>Shc</sup>-expressing MCF7 cells did not change EGF-induced proliferation or viability. These data suggest that EGF-induced MAPK activation in epithelial cells is ensured by a redundant coupling of Grb2 to the receptor.

In a quest for growth factor-independent pathways involving Shc-mediated Erk activation, we investigated signaling downstream of the cell-cell adhesion molecule E-cadherin. We identified a previously unknown signaling pathway which is induced upon disruption of E-cadherin-dependent cell-cell adhesion. This pathway involves Src- and Shc-dependent Erk activation, which results subsequently in the expression of the urokinase plasminogen activator. Applying the knockdown-in technique revealed that p46<sup>Shc</sup> and p52<sup>Shc</sup>, but not p66<sup>Shc</sup>, were able to mediate MAPK activation upon disruption of cell-cell adhesion. This pathway directly links disruption of cell-cell adhesion with the expression of proteolytic enzymes, both processes involved in metastasis and wound healing.

To learn more about the role of p66<sup>Shc</sup> in mediating oxidative stress-induced apoptosis in epithelial cells, the effect of p66<sup>Shc</sup> on cell viability was investigated. Although p66<sup>Shc</sup> has been shown to enhance stress-induced apoptosis in fibroblasts, endothelial cells, and T-cells, no effect on p66<sup>Shc</sup> expression was observed in two different epithelial cells, suggesting that the apoptotic response in epithelial cells is mediated in a p66<sup>Shc</sup>-independent manner.

# 1. INTRODUCTION

This chapter provides insights into three different topics: (i) function of Shc proteins, (ii) E-cadherin-mediated cell-cell adhesion and (iii) RNA interference.

## 1.1 The Shc adaptor proteins

Shc proteins are prototype adaptor proteins which represent molecules that possess no apparent catalytic domains or activities. Adaptor proteins contain modular protein-protein and protein-lipid interaction domains, such as src-homology domain 2 (SH2) and 3 (SH3), phosphotyrosine binding domain (PTB), and pleckstrin homology (PH) domains, and are essential in propagating signals from a receptor in a coordinated fashion (Zhang et al., 2002).

The adaptor protein ShcA was initially identified as an SH2-containing proto-oncogene involved in growth factor signaling. Since then, it has been shown to be an integral component implicated in the action of a wide variety of receptors, including receptor tyrosine kinases (RTKs), G protein-coupled receptors (GPCRs), immunoglobulin receptors, and integrins, as well as non-receptor tyrosine kinases such as Src and FAK. To date, three mammalian *shc* genes have been identified: *shcA*, *shcB* (*sck*), and *shcC* (*N-shc/rai*) (Nakamura et al., 1996; O'Bryan et al., 1996; Pelicci et al., 1996). All three *shc* genes encode proteins that are highly related in domain and structure. In the following section, I will provide an overview of the genomic organization and structural architecture of ShcA, hereafter referred to as Shc, along with its known functions in signal transduction.

### 1.1.1. Genomic and structural organization of Shc

#### 1.1.1.1 Genomic organization and regulation of Shc expression

The human *shc* locus maps to the chromosome 1q21 (Huebner et al., 1994). It contains 13 exons, which give rise to three different gene products: three isoforms of about 46, 52, and 66 kDa. All isoforms are generated either through RNA splicing or alternative translational initiation (Migliaccio et al., 1997; Pelicci et al., 1992) (Fig. 1.1.1.1). While the p46<sup>Shc</sup>/p52<sup>Shc</sup> transcript originates from the assembly of the non-coding exon 1 with the 3' portion of exon 2 (exon 2a), and with exons 3–13, the p66<sup>Shc</sup> transcript is formed by the assembly of exons 2–13. A second mechanism that regulates transcription of the three Shc isoforms is the alternative usage of in-frame translational start codons. The transcript encoding p66<sup>Shc</sup> has three in-frame ATGs that are responsible for the translation of p66<sup>Shc</sup>, and, to a lesser extent, p52<sup>Shc</sup>, and p46<sup>Shc</sup>. The p52<sup>Shc</sup>/p46<sup>Shc</sup> transcript contains two in-frame ATGs that are responsible for the translation of p52<sup>Shc</sup> and p46<sup>Shc</sup> (Migliaccio et al., 1997). The mouse *shc* locus is similarly organized and maps to chromosome 3 (Kojima et al., 2001; Migliaccio et al., 1997).

Less is known about the molecular mechanisms that regulate the differential expression of the various Shc isoforms. It seems that different mechanisms control the expression of the two main Shc transcripts in different cell types. p46<sup>Shc</sup>/p52<sup>Shc</sup> are found

ubiquitously in every cell type, whereas  $p66^{Shc}$  expression varies and is restricted to certain tissues and cell lines, being absent in brain, in most hematopoietic cell lines, in peripheral blood lymphocytes (PBL), and in a subset of breast cancer cell lines (Jackson et al., 2000; Pelicci et al., 1992; Stevenson and Frackelton, 1998; Xie and Hung, 1996). Ventura et al. (Ventura et al., 2002) have recently identified epigenetic modifications, namely histone deacetylation and cytosine methylation, as mechanisms underlying transcriptional silencing of  $p66^{Shc}$  in specific cell types. Histone deacetylase inhibitors, or demethylating agents, were capable of restoring  $p66^{Shc}$  expression in primary, immortalized, and transformed cells. Additionally, the  $p66^{Shc}$ -encoding locus could be reactivated in human PBL and mouse T-cells by treatment with a variety of apoptogenic stimuli, such as  $H_2O_2$ , the calcium ionophore A23187, Fas ligation, and sequential

engagement of CD4 and CD3 (Pacini et al., 2004). In vivo,  $p66^{Shc}$  expression has been found to be induced in circulating peripheral blood mononuclear cells of diabetic patients (Pagnin et al., 2005).

Overall expression analysis has shown that Shc is expressed at its highest levels in the placenta, adipocytes, bronchial-epithelial cells, colorectal adenocarcinoma, cardiac myocytes, and smooth muscle cells of humans (human GNF SymAtlas).

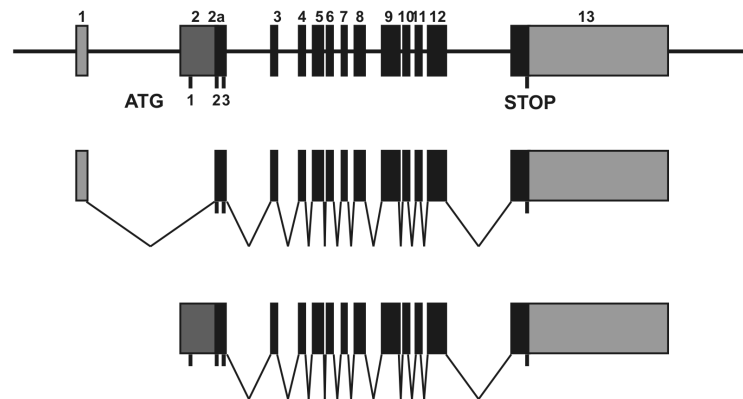
The family members ShcB and ShcC are derived from different genes, and their expression is restricted to the brain and neuronal tissue (Nakamura et al., 1996; O'Bryan et al., 1996; Ponti et al., 2005). Unlike *shcA*, only two isoforms are encoded by the *shcB* and *shcC* loci.

#### Figure 1.1.1.1:

##### Organization of human *Shc* locus and exon assembly of *Shc* transcripts.

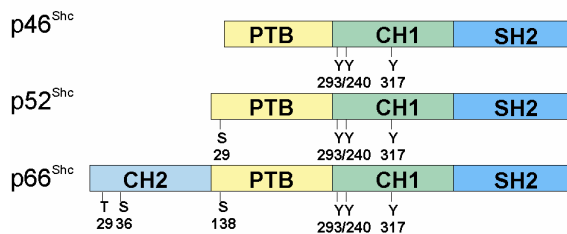
A schematic representation of the exon assembly in the  $p52^{Shc}/p46^{Shc}$  and  $p66^{Shc}$  encoding transcripts. *Shc* exons are indicated by

boxes (black boxes are translated exons), the exon numbers are given above, and the splicing events are shown by the zig-zag line. The position of the three *Shc* ATGs is indicated below the exons (as described in (Migliaccio et al., 1997)).



### 1.1.1.2 Structural organization of Shc proteins

Shc proteins are characterized by their specific modular organization, consisting of an amino-terminal phosphotyrosine-binding (PTB) domain, a central proline- and glycine-rich collagen homology domain (CH1), and a carboxy-terminal Src homology 2 (SH2) domain (Fig. 1.1.1.2). The unique feature thereby is the arrangement of the PTB and the SH2 domain in an N to C order (Luzi et al., 2000). Shc proteins are evolutionarily well conserved and can be found in mammals, fishes, flies and worms.



**Figure 1.1.1.2: Domain structure of Shc proteins.** All Shc isoforms share the same modular organization: N-terminal PTB domain, central collagen homology domain (CH1), and C-terminal SH2 domain. p66<sup>Shc</sup> contains an additional collagen homology domain (CH2). All known phosphorylation sites are indicated.

A second phosphotyrosine-binding (PTB) domain, distinct from the SH2 domain, was discovered in Shc proteins (Blaikie et al., 1994; Kavanaugh and Williams, 1994). The unique feature of the Shc-PTB domain is that its binding to target sequences is determined by residues N-terminal to the phosphotyrosine, and is not influenced by residues C-terminal to the phosphotyrosine (Blaikie et al., 1997; Trub et al., 1995; Zhou et al., 1995a). Today, more than 160 proteins containing a PTB domain are known, including insulin receptor substrate 1/2

(IRS-1/2), tensin, the epidermal growth factor receptor (EGFR) pathway substrate (Eps8), and the integrin cytoplasmic domain-associated protein-1 (ICAP-1) (Schlessinger and Lemmon, 2003).

The PTB domain shows remarkable structural similarity to pleckstrin homology (PH) domains, despite a very divergent primary sequence (Zhou et al., 1995c). In a similar way to PH domains, the Shc-PTB domain has been shown to bind acidic phospholipids such as PI(4,5)P<sub>2</sub> and PI(4)P (Zhou et al., 1995c), and also PI(3,4,5)P<sub>3</sub> (Rameh et al., 1997). The high affinity ( $K_D=10-50 \mu\text{M}$ ) of this binding suggests that the interaction of Shc with the membrane could occur independently of an interaction with tyrosine-phosphorylated receptors. Consistent with this idea was the identification of residues within the Shc-PTB domain that are critical for phospholipid binding and membrane localization and are distinct from the residues necessary for phosphotyrosine binding (receptor binding). Over the last few years many different proteins, such as F-actin, SHIP (SH2-containing inositol polyphosphate 5 phosphatase), IRS-1 and PP2A (protein phosphatase type 2A), have been found to bind to the Shc-PTB domain in a phosphotyrosine-dependent or -independent manner (Kasus-Jacobi et al., 1997; Lamkin et al., 1997; Thomas et al., 1995; Ugi et al., 2002).

On the N-terminal edge of the PTB domain of p52<sup>Shc</sup> and p66<sup>Shc</sup> there is a serine phosphorylation site (Fig. 1.1.1.2) (El-Shemerly et al., 1997). Further studies have demonstrated that phosphorylation of this site is necessary for Shc binding to the phosphatase PTP-PEST and downregulation of insulin-induced Erk activation, most likely



through dephosphorylation of Shc (Faisal et al., 2002).

The SH2 domain of Shc is located at the C-terminus and was thought to be the only domain responsible for the recruitment of Shc to activated growth factor receptors before the identification of the Shc-PTB domain. It folds in a very similar manner to other SH2 domains (Mikol et al., 1995; Zhou et al., 1995b). Unlike the Shc-PTB domain, the target binding of the Shc-SH2 domain is determined by residues C-terminal to the phosphotyrosine (Ravichandran, 2001).

Between the PTB and the SH2 domain is the collagen homology (CH) 1 domain. This region is characterized by a large number of glycine and proline residues, but does not feature typical collagen-like repeats. While the PTB and the SH2 domains share high similarity, 78% and 68% respectively, the CH1 domain is generally less well conserved between different species. However, within the mammalian Shc family members, three regions sharing a higher degree in homology are present in this domain. Two of these conserved regions comprise three critical tyrosine phosphorylation sites, Y239, Y240, and Y317, and additional amino acids surrounding the amino-terminal phosphorylation site suggesting an important role in the recognition of effector proteins (O'Bryan et al., 1996). Y317 is conserved in mammalian Shc proteins, but not seen in those of lower organisms. Y239 and Y240 are also present in *Drosophila* Shc (Lai et al., 1995), but Shc in *C. elegans* does not contain any of the tyrosine residues (Luzi et al., 2000). Both phosphorylation sites conform to the consensus Grb2-binding site and have been demonstrated to bind Grb2 (Velazquez et al., 2000; Walk et al., 1998).

The third conserved region maps as a binding site for adaptins which links the endocytic machinery of clathrin-coated pits with integral membrane proteins, suggesting a potential role of Shc in endocytosis. This region is only weakly conserved in *Drosophila* (Lai et al., 1995).

p66<sup>Shc</sup> contains an additional N-terminal CH-like domain (called CH2) (Migliaccio et al., 1997), which is also found in the longer isoforms of ShcB and ShcC, but not in the *Drosophila* Shc protein (Luzi et al., 2000). In contrast to the CH1 domain, the CH2 domain can be serine/threonine phosphorylated in response to several stimuli such as oxidative stress (Migliaccio et al., 1999), 12-O-tetradecanoylphorbol-13 acetate (TPA) (El-Shemerly et al., 1997), and epidermal growth factor (EGF) (Okada et al., 1997). The phosphorylation of serine 36 (S36) has been linked to the role of p66<sup>Shc</sup> in oxidative stress response (Migliaccio et al., 1999) and will be discussed later. The physiological relevance of the threonine phosphorylation site (T29) has not yet been defined.

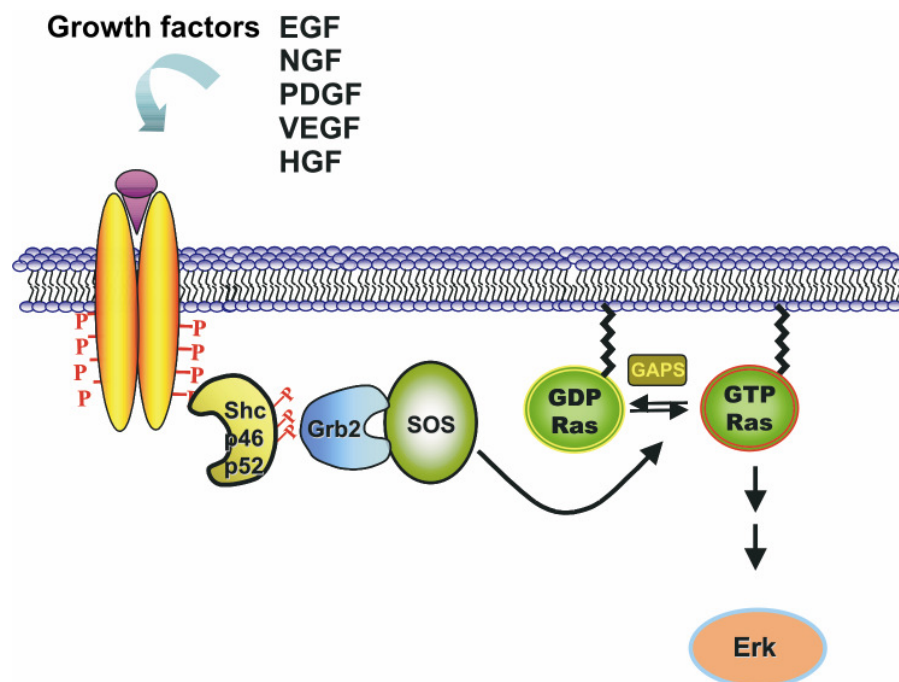
## 1.1.2 Signaling and function of ShcA

### 1.1.2.1 Role of Shc in mitogenic Ras/Erk signaling

In vivo and in vitro studies from various laboratories have clearly established a role for Shc in Ras/MAPK activation (Lai and Pawson, 2000; Pratt et al., 1999; Salcini et al., 1994). This is the only function of Shc of which the molecular mechanism is understood. Activation of RTKs results in the recruitment of Shc proteins and, subsequently, in Shc phosphorylation. Phosphorylated, hence activated, Shc binds to the Grb2/SOS complex.

The Shc/Grb2/SOS complex is then localized to the membrane through the interaction of Shc with the phosphorylated receptor via its PTB or SH2 domain (Blaikie et al., 1994; Pelicci et al., 1992; Ravichandran et al., 1993). At the membrane in vicinity to Ras, SOS stimulates nucleotide exchange on Ras and, thereby, activation of Ras (Fig. 1.1.2.1) (Ravichandran, 2001). GPCR, integrins, and cytokine receptors without intrinsic tyrosine kinase activity utilize other soluble and associated tyrosine kinases to phosphorylate Shc (Sayeski and Ali, 2003; Velazquez et al., 2000; Wary et al., 1996). In addition to translocating the Grb/SOS complex to the membrane, Shc seems to influence the extent of Ras activation. The Shc/Grb2 interaction increases the level of SOS bound to Grb2 in some systems, and SOS has been found

preferentially in complexes that also contain Shc (Buday et al., 1995; Pronk et al., 1994; Ravichandran et al., 1995). Still, many receptors are able to directly recruit the Grb2/SOS complex, leading to Ras activation without the involvement of Shc (Arvidsson et al., 1994; Batzer et al., 1994; Schlaepfer et al., 1998). In response to integrin ligation, however, Shc is necessary and sufficient for activation of the MAP kinase pathway (Wary et al., 1996). The ability of Shc to mediate Ras activation is largely dependent on the three tyrosine residues within its CH1 domain. Phosphorylation-deficient mutants exert dominant-negative activity, whereby the importance of distinct Shc tyrosines differs between the cell types and receptors (Ravichandran, 2001).



**Figure 1.1.2.1: Model for Shc-mediated Ras activation downstream of RTK.** Shc binds to RTKs and recruits the Grb2/SOS complex which activates Ras. See text for details.

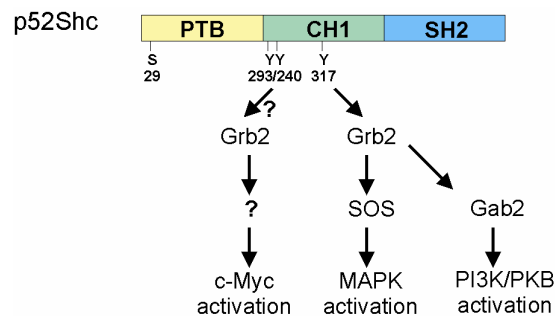
### 1.1.2.2 Role of Shc in c-myc activation and cell survival

The observation that Shc is involved in *c-myc* activation has led to two suggestions. First, Shc might play a role in signaling other than mediating Ras/MAPK activation and, second, the downstream signaling of Y239/Y240 and Y314 might have distinct properties (Fig. 1.1.2.2). In BaF cells, Gotoh et al. (Gotoh et al., 1996) showed that Shc could induce *c-myc* expression in response to IL-3 stimulation which was dependent on Y239/Y240, but not on Y137. The same situation was demonstrated for EGF signaling in NIH3T3 cells (Gotoh et al., 1997). Subsequently, a role for Shc in *c-myc* gene activation has been shown in IL-2 signaling (Lord et al., 1998), in PDGF signaling (Blake et al., 2000), and in T-cell antigen receptor (TCR) signaling (Patrussi et al., 2005). However, it remains unclear how Shc mediates *c-myc* activation and what target genes are in turn affected by c-Myc.

Induced *c-myc* expression downstream of IL-2/3 and TCR correlated with survival signals in hematopoietic cells (Gotoh et al., 1996; Lord et al., 1998; Patrussi et al., 2005), suggesting an involvement of Shc in the regulation of a pro-survival pathway via *c-myc*. Lord et al. (Lord et al., 1998) observed Shc-dependent induction of proliferation and expression of *c-myc*, *bcl-2* and *bcl-x* in response to IL-2. Nevertheless, the proliferative response and the expression of *bcl*-family genes were not sufficient to mediate sustained cell survival and antiapoptotic effects associated with a complete IL-2 signal in murine T-cells. In a different study, a Shc chimera fused to the IL-2 receptor  $\beta$  chain that lacks other cytoplasmic tyrosines was able to evoke PKB/AKT

phosphorylation via the Shc/Grb2/Gab2/PI3K pathway, and might therefore be involved in the regulation of IL-2-mediated cell survival (Fig. 1.1.2.2) (Gu et al., 2000).

The involvement of ShcB and ShcC in survival of neuronal cells has become more evident. Whereas ShcA is only expressed in proliferating neuroblasts and is downregulated in post-mitotic neurons, ShcB and ShcC remain expressed (Cattaneo and Pelicci, 1998; Conti et al., 1997). Mice with no ShcB and/or ShcC expression display a loss of certain types of peptidergic and nociceptive neurons (Sakai et al., 2000). It appears, therefore, that ShcA plays a role in neuronal proliferation, but ShcB and ShcC isoforms play a role in survival of post-mitotic neurons.



**Figure 1.1.2.2: Distinct signaling capacities of the major tyrosine phosphorylation sites.** The three tyrosine phosphorylation sites and the signaling linked to these tyrosines are indicated.

### 1.1.2.3 Role of Shc in cell adhesion, migration, and cytoskeletal organization

The implication of Shc in processes such as cell adhesion, migration, and cytoskeletal organization originates from diverse reports in different contexts.

Embryonic fibroblasts derived from Shc-knockout mice have defects in spreading on fibronectin (Lai and Pawson, 2000). Similarly,

the regulation of cell adhesion and EGF-induced migration on fibronectin required the interaction of Shc and  $\alpha 5 \beta 1$  integrin in MCF7 breast cancer cells (Mauro et al., 1999; Nolan et al., 1997). In addition, Shc has been shown to localize to focal adhesions and to interact with the focal adhesion kinase (FAK) (Barberis et al., 2000; Gu et al., 1999). Although Shc can be a substrate of FAK (Schlaepfer et al., 1998), their effects on cell migration seem to be distinct. While Shc stimulates random cell motility through activation of the Erk signaling pathway, FAK regulates directional persistent migration via p130Cas (Gu et al., 1999). In ErbB2-driven migration, Shc seems to be required for lamellipodia formation (reorganization of the actin cytoskeleton) and for mediating the interaction between the receptor and Memo, which is necessary for cell migration-required reorganization of the microtubule network (Marone et al., 2004). In support of this report, inhibition of EGF-induced cell migration upon downregulation of Shc has also been observed in a different study (Nolan et al., 1997). In response to HGF, overexpression of Shc enabled enhanced migration and growth of melanoma cells (Pelicci et al., 1995a). Whether Shc stimulates proliferation or migration seems, at least partially, to be determined by external stimuli. In the presence of growth factors, Shc regulates DNA synthesis, but under growth factor-limiting conditions, Shc stimulates cell migration (Collins et al., 1999). To what extent both responses depend on Shc-induced MAPK activation, or activation of and cross talk with other signaling pathways, is not clear. However, in one case, a direct interaction between Shc and F-actin has been observed in PC12 cells in response to NGF (Thomas et al., 1995).

#### 1.1.2.4 Role of Shc in tumorigenesis

The ability of Shc to mediate mitogenic signaling raises the question of whether Shc can drive tumorigenesis. Although Shc proteins do not contain any enzymatic activity, overexpression of p46/52<sup>Shc</sup> was able to transform mouse fibroblasts and to enable them to form tumors in nude mice (Pelicci et al., 1992). In tumor cells with known tyrosine kinase gene alteration, Shc proteins were found to be constitutively phosphorylated and complexed with Grb2 and activated tyrosine kinases (EGFR, PDGFR, ErbB-2, Met, BCR-Abl, and Ret) (Pelicci et al., 1995b). Underscoring the role of Shc in oncogenic RTK signaling, dominant negative Shc has been shown to block proliferation of ErbB-2 positive human breast cancer cell lines (Stevenson et al., 1999).

More recently, an *in vivo* study has unveiled an unsuspected role for the Shc in RTK-mediated vascular endothelial growth factor (VEGF) production and tumor angiogenesis (Saucier et al., 2004). Using RTK engineered to recruit a defined signaling protein, it was shown that the direct recruitment of either Grb2 or Shc to an RTK oncoprotein is sufficient to induce transformation and metastasis (Saucier et al., 2002). The authors then extended this study in order to compare and define the role of Shc and Grb2 in RTK oncoprotein-driven tumorigenesis (Saucier et al., 2004). Fibroblasts expressing Shc-binding RTK oncoproteins induced tumors with short latency (approximately 7 days), whereas cells expressing Grb2-binding RTK oncoproteins induced tumors with delayed latency (approximately 24 days). The early onset of tumor formation resulted in the ability of Shc-binding RTK oncoproteins to produce (VEGF)

in culture and an angiogenic response *in vivo*. Moreover, the use of fibroblasts derived from Shc-deficient mouse embryos demonstrated that Shc was essential for the induction of VEGF by the Met/hepatocyte growth factor RTK oncoprotein and by serum-derived growth factors.

### 1.1.2.5 *In vivo* function of Shc

#### 1.1.2.5.1 *Conventional Shc knockout*

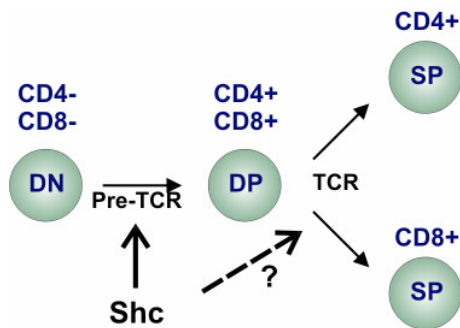
The conventional knockout mouse created by Lai and Pawson (Lai and Pawson, 2000) clearly established a role for Shc *in vivo*. Ablation of exons 2 and 3, which encode the PTB domain, by gene targeting resulted in a loss of expression of all three Shc isoforms in homozygous mutants. The homozygous mutant embryos died at day 11.5 with severe defects in heart development and establishment of mature blood vessels. The cardiovascular system showed defects in angiogenesis and cell-cell contacts. Consistent with this, Shc was mainly expressed in the cardiovascular system of wild-type embryos. The Shc<sup>Δex2/3</sup> mutants also provided evidence for Shc in MAPK signaling *in vivo*. There was a loss of MAPK activation within the cardiovascular system of the Shc<sup>Δex2/3</sup> mutants, as revealed by whole mount immunostaining with phospho-specific Erk antibodies, when compared to wild-type embryos. Studies with Shc<sup>Δex2/3</sup> embryonic fibroblasts have demonstrated that Shc is necessary for MAPK signaling induced by a low concentration of growth factors, but at a high concentration of growth factors (50 ng/ml EGF or 25 ng/ml PDGF) no detectable difference in MAPK activation was observed. These data suggest that Shc sensitizes cells to low amounts of

growth factors. Shc-deficient mouse embryonic fibroblasts (MEFs) also showed changes in focal contact organization and actin stress fibers when plated on fibronectin, underscoring the role of Shc in cytoskeletal organization.

#### 1.1.2.5.2 *Conditional T-cell specific knockout and transgenic mice*

Efforts over the past 10 years have demonstrated that Shc plays a critical role in T-cell receptor (TCR) signaling. The earliest evidence linking Shc to TCR-mediated signaling was the observation that Shc becomes tyrosine phosphorylated rapidly after TCR/CD3 crosslinking (Ravichandran et al., 1993). Several studies followed showing that expression of dominant negative mutants of Shc inhibited TCR-mediated downstream signaling (Milia et al., 1996; Pacini et al., 1998; Pratt et al., 1999). To examine the relative significance of Shc compared to several other adaptors in T-cells, two genetic approaches were taken in mice (Zhang et al., 2002). The first approach involved the generation of a transgenic mouse with thymocyte-specific expression of a dominant negative form of Shc, where all tyrosine residues were mutated to phenylalanine (ShcFFF). The ShcFFF transgenic mice had a reduced thymus size, with significant reduction in thymocyte numbers. Further analysis revealed that T-cells<sup>ShcFFF</sup> were blocked at the double negative stage (DN) of their development, which was characterized by the absence of CD4 and CD8 markers (reviewed in (Zhang et al., 2003)). The authors did not observe any increase in the apoptotic fraction of the DN cells in ShcFFF transgenic mice compared to wild-type mice. More recent studies using pulse BrdU injection have demonstrated a defect in proliferation of

the late DN stage cells mediated by the pre-TCR (Fig. 1.1.2.5.2). The same phenotype was also obtained using the second approach, conditional Shc knockout mice, with a nearly complete loss of Shc protein expression in thymocytes. Thus, both Shc expression and its tyrosine phosphorylation play an essential and non-redundant role in thymic T-cell development and proliferation.

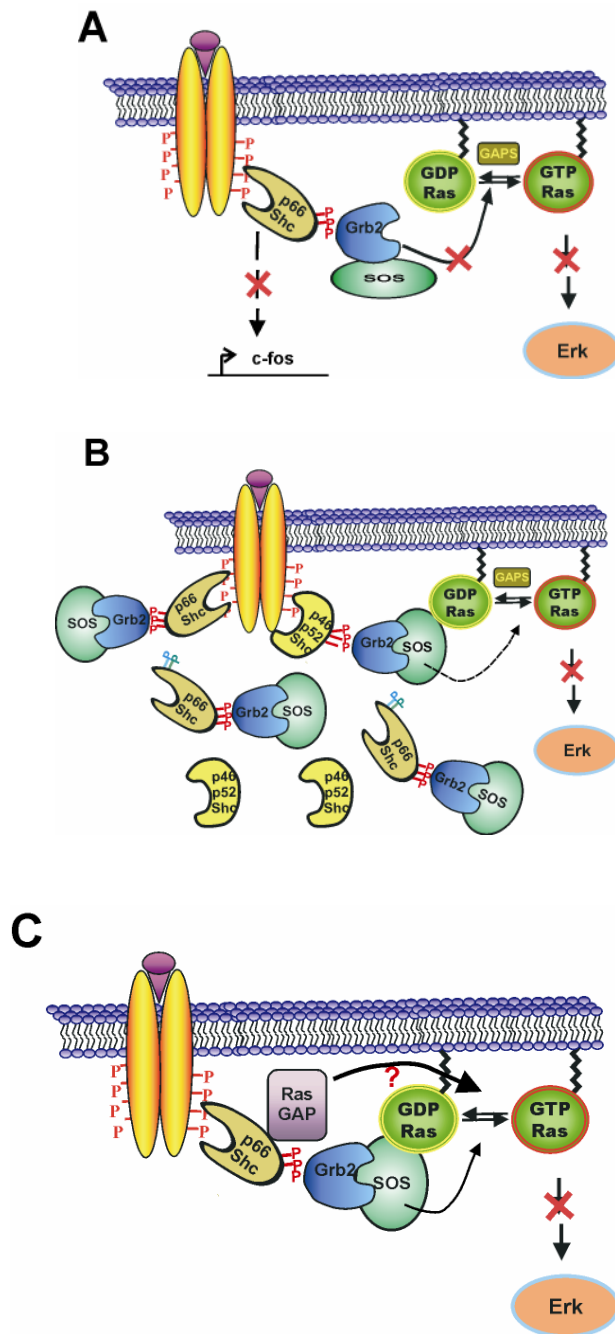


**Figure 1.1.2.5.2: Role of Shc in T-cell development.** Inducible expression of *ShcFFF* as a transgene or inducible loss of Shc protein expression arrests thymic development at the double negative (DN) stage. The block is seen where signaling from the pre-TCR occurs. The role of Shc during selection at the double positive (DP) stage has not yet been determined. SP: single positive; CD4 and CD8 are T-cell markers (adapted from (Zhang et al., 2003)).

#### 1.1.2.6 Role of p66<sup>Shc</sup>

The cDNA encoding the largest isoform, p66<sup>Shc</sup>, was cloned in 1997, 5 years after the discovery of the two smaller isoforms (Migliaccio et al., 1997). As already mentioned, it encompasses an additional CH2 domain on its N-terminus containing a serine (S36) and threonine (T29) phosphorylation site. Unlike p46/52<sup>Shc</sup>, overexpression of p66<sup>Shc</sup> does not

transform mouse fibroblasts (Migliaccio et al., 1997), suggesting a function distinct from the other two isoforms. Indeed, p66<sup>Shc</sup> does not increase EGF-induced MAPK activation, although it is tyrosine-phosphorylated upon EGF stimulation, binds to activated EGFRs, and forms stable complexes with Grb2 (Migliaccio et al., 1997) (Fig. 1.1.2.6-1A). Furthermore, it has been shown that p66<sup>Shc</sup> expression inhibits EGF-induced *c-fos* promoter activation (Fig. 1.1.2.6-1A). The molecular mechanism is not understood, taken into account that p66<sup>Shc</sup> expression did not inhibit Erk activation. However, the inhibition was attributed to the CH2 domain, since it retained the inhibitory effect of p66<sup>Shc</sup> on the *c-fos* promoter (Migliaccio et al., 1997). In contrast, an independent study has shown that p66<sup>Shc</sup> can function in a dominant-interfering manner and inhibits Erk activation downstream of EGFR signaling (Fig. 1.1.2.6-1B) (Okada et al., 1997). These authors demonstrated not only tyrosine but also serine/threonine phosphorylation of p66<sup>Shc</sup> in response to EGF, which impairs its ability to associate with the tyrosine-phosphorylated EGFR, but not with Grb2. Co-immunoprecipitation of Shc and Grb2 from cells overexpressing the p45/52<sup>Shc</sup> isoforms, versus p66<sup>Shc</sup>, directly demonstrated a competition of binding for a limited pool of Grb2 proteins (Fig. 1.1.2.6-1B). Inhibition of the Ras/MAPK pathway by p66<sup>Shc</sup> in an S36 phosphorylation-dependent manner has also been found following TCR downstream signaling (Pacini et al., 2004). Furthermore, p66<sup>Shc</sup>-deficient T-cells have been reported to proliferate faster than their normal counterparts in response to limiting ligand concentration, supporting an antagonistic activity of p66<sup>Shc</sup> on mitogenic signaling (Pacini et al., 2004). The



**Figure 1.1.2.6-1: Possible mechanism of p66<sup>Shc</sup> function in Ras/MAPK signaling.** See text for details (A) p66<sup>Shc</sup> binds Grb2 in a conformation which does not allow activation of Ras. (B) p66<sup>Shc</sup> competes with p46/p52<sup>Shc</sup> for Grb2 binding. (C) p66<sup>Shc</sup> binds to RasGAP and negatively influences Ras activation.

mechanism whereby p66<sup>Shc</sup>-bound Grb2 becomes uncoupled from Ras remains to be determined. It is possible that p66<sup>Shc</sup> binds Grb2 or the Grb2/SOS complex in a conformation which does not allow SOS to act as a guanine exchange factor for Ras (Fig. 1.1.2.6-1A). However, the finding that p66<sup>Shc</sup> participates in a complex which also includes RasGAP during early morphogenetic events in *Xenopus* gastrulation (Dupont and Blancq, 1999) suggests a different mechanism for the negative control of Ras/MAPK activation by this protein (Fig. 1.1.2.6-1C). Whatever the mechanism is, p66<sup>Shc</sup> does not mediate growth factor-induced MAPK activation, and its expression might provide a mechanism for fine-tuning the Ras/MAPK pathway.

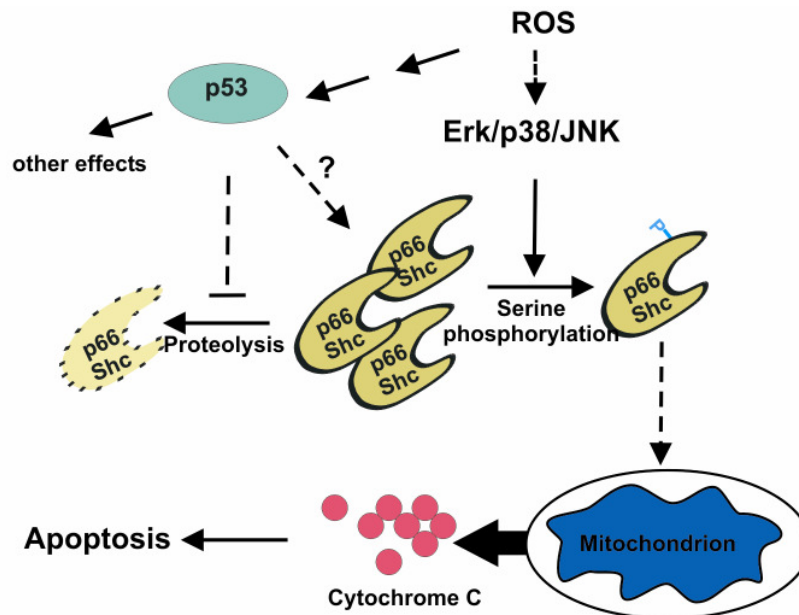
More recently, loss-of-function studies have unveiled an unexpected role of p66<sup>Shc</sup> in ageing and in the apoptotic response to oxidative stress (Migliaccio et al., 1999). p66<sup>Shc</sup>-deficient mice exhibit a lifespan about 30% longer than wild-type. Moreover, they survive longer after treatment with paraquat, a drug that increases the production of reactive oxygen species (ROS) and, therefore, oxidative stress. Increased resistance to oxidative stress or oxidative stress-inducing agents such as UV and H<sub>2</sub>O<sub>2</sub> can be correlated with a reduction in the apoptotic responses to these stimuli in p66<sup>Shc</sup>-/- fibroblasts. A protective effect of p66<sup>Shc</sup> ablation against apoptosis in thymocyte and peripheral T-lymphocyte has also been reported recently (Pacini et al., 2004). Conversely, p66<sup>Shc</sup> overexpression results in enhanced stress-induced apoptosis in fibroblasts, endothelial cells and T-cells (Pacini et al., 2004; Trinei et al., 2002). The proapoptotic activity of p66<sup>Shc</sup> is strictly dependent on phosphorylation of S36 in the CH2 domain. S36 phosphorylation is



observed in response to many stimuli, including  $H_2O_2$ , UV (Migliaccio et al., 1999), Fas ligation (Pacini et al., 2004), and taxol (Yang and Horwitz, 2002), but also in response to EGF (Okada et al., 1997) and insulin (Kao et al., 1997). Depending on the cellular context and on the identity of the stimulus, either Erk, JNK, or p38 MAPK is responsible for S36 phosphorylation (Le et al., 2001; Okada et al., 1997; Yang and Horwitz, 2002). Taken together, these results suggest that  $p66^{Shc}$  acts as a sensor of intracellular concentration of ROS (Fig. 1.1.2.6-2).

Further experiments aimed at understanding the mechanisms underlying the role of  $p66^{Shc}$  in regulating oxidative stress-induced apoptosis have revealed that  $p66^{Shc}$  is a downstream effector of the tumor suppressor p53 (Trinei et al., 2002). It is required for p53-induced release of cytochrome C from

mitochondria, and subsequent caspase 3 activation (Fig. 1.1.2.6-2). Again, the capacity of  $p66^{Shc}$  to mediate p53-dependent apoptosis requires phosphorylation of S36. The release of cytochrome C in oxidative stress is the endpoint of the p53-dependent transcriptional activation of redox related genes. The resulting rise of ROS levels affects the mitochondrial membrane potential, leading to membrane permeability transition and cytochrome C release (Li et al., 1999; Polyak et al., 1997). Cyclosporin A, an inhibitor of the mitochondrial permeability transition pore which blocks oxidative stress-induced apoptosis of wild-type MEFs, is able to prevent re-expressed  $p66^{Shc}$  from restoring apoptotic responses to oxidants in  $p66^{Shc-/-}$  MEFs, suggesting that  $p66^{Shc}$  may regulate mitochondrial permeability transition, and



**Figure 1.1.2.6-2:  $p66^{Shc}$  senses ROS and mediates oxidative stress-induced apoptosis.** ROS activate one of the MAPKs, which in turn phosphorylates  $p66^{Shc}$  on S36. S36 phosphorylation is necessary for cytochrome C release and subsequent apoptosis. p53 acts upstream of  $p66^{Shc}$  and enhances  $p66^{Shc}$  protein stability, leading to  $p66^{Shc}$  accumulation. p53-induced apoptosis is dependent on  $p66^{Shc}$  expression.

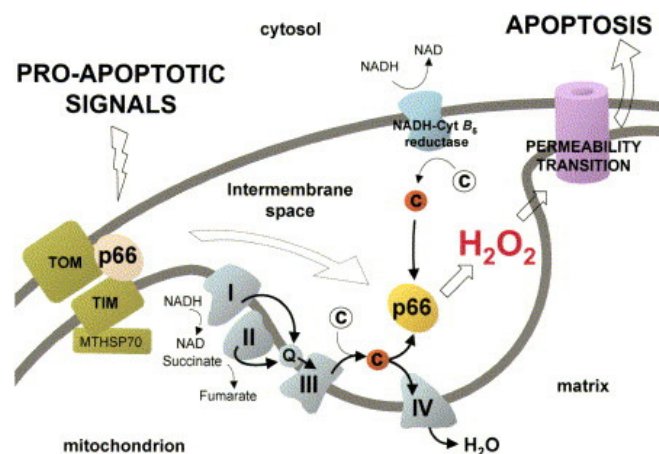


hence cytochrome C release, by modulating the production of ROS (Orsini et al., 2004). Indeed, intracellular ROS levels are drastically reduced in p66<sup>Shc-/-</sup> cells and enhanced in p66<sup>Shc</sup> overexpressing cells (Nemoto and Finkel, 2002; Orsini et al., 2004). Furthermore, p66<sup>Shc</sup> has been found to localize to mitochondria and to be associated with Hsp70. (Orsini et al., 2004). The best evidence was derived from a recent report by Giorgio *et al.* (Giorgio et al., 2005), which clearly established a role for p66<sup>Shc</sup> in the generation of ROS. p66<sup>Shc</sup> was found to function as a redox enzyme that generates mitochondrial ROS as signaling molecules for apoptosis (Fig. 1.1.2.6-3). It does so by utilizing reducing equivalents of the mitochondrial electron transfer chain through the oxidation of cytochrome C. Interestingly, S36 phosphorylation was not observed in the mitochondrial pool of p66<sup>Shc</sup>: instead a different region was necessary for the redox activity of p66<sup>Shc</sup>. It seems, therefore, that p66<sup>Shc</sup> exists in two different pools, a cytoplasmic one and a mitochondrial one. Significant translocation of p66<sup>Shc</sup> from cytosol to mitochondria does not occur following

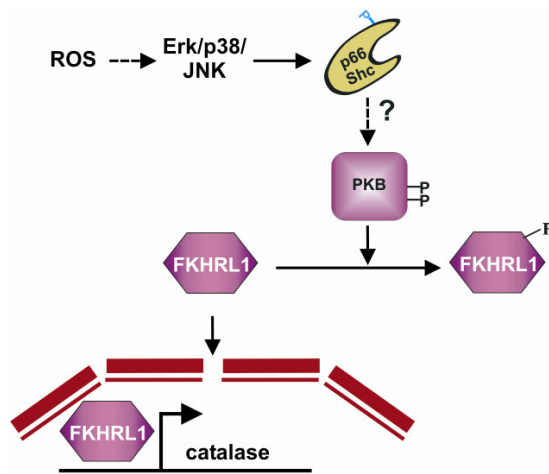
apoptotic signals, suggesting that S36 phosphorylation might serve other, nonmitochondrial, activities of p66<sup>Shc</sup> which are also needed to exert its proapoptotic function.

A second mechanism by which p66<sup>Shc</sup> could influence ROS levels was suggested by Nemoto *et al.* (Nemoto and Finkel, 2002) (Fig. 1.1.2.6-4). They linked p66<sup>Shc</sup> expression to the transcriptional activity of the forkhead family transcription factor, FKHRL1. In quiescent cells, FKHRL1 localizes predominantly in the nucleus where it positively regulates transcription of genes such as catalase, implicated in ROS scavenging. Oxidative stress most probably promotes FKHRL phosphorylation in a PKB-dependent manner, and subsequent exclusion from the nucleus results in a reduction of its transcriptional activity. Phosphorylation and cytoplasmic localization of FKHRL in response to H<sub>2</sub>O<sub>2</sub> was abrogated in p66<sup>Shc</sup>-deficient MEFs. Accordingly, FKHRL-dependent transcription of the catalase gene was augmented in these cells, suggesting a pivotal role of p66<sup>Shc</sup> in the

**Figure 1.1.2.6-3: Model of p66<sup>Shc</sup> redox activity during mitochondrial apoptosis.** Proapoptotic signals induce release of p66<sup>Shc</sup> from a putative inhibitory complex. Active p66<sup>Shc</sup> then oxidizes reduced cytochrome C (red) and catalyzes the reduction of O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub>. Permeability transition pore opening by H<sub>2</sub>O<sub>2</sub> then leads to swelling and apoptosis. NADH-Cyt B<sub>5</sub> reductase is indicated as an additional putative source of reduced cytochrome C (taken from (Giorgio et al., 2005)).



redox-dependent inactivation of FKHL1 and, thereby, in the control of ROS.



**Figure 1.1.2.6-4:  $p66^{Shc}$  regulates FKHL1 transcriptional activity.**  $p66^{Shc}$  expression enhances PKB phosphorylation via an unknown mechanism. This leads to a decrease in FKHL1 transcriptional activity due to phosphorylation by PKB which causes its retention in the cytoplasm. Finally, ROS-detoxifying enzymes such as catalase are less expressed.

The ability to generate ROS and to regulate expression of scavenger proteins makes  $p66^{Shc}$  an attractive target for therapies against vascular diseases, which are strongly mediated by ROS. Indeed, deletion of  $p66^{Shc}$  reduces systemic and tissue oxidative stress, vascular cell apoptosis and early atherogenesis in mice fed a high-fat diet (Napoli et al., 2003).  $p66^{Shc}$ -deficient mice were also resistant to the proapoptotic/hypertrophic action of Angiotensin II (Ang II). Consistently, *in vitro* experiments have shown that Ang II causes a higher rate of apoptotic death in cardiomyocytes isolated from  $p66^{Shc(+/+)}$  hearts than in those isolated from  $p66^{Shc(-/-)}$  hearts (Graiani et al., 2005). In

perspective, inhibition of  $p66^{Shc}$  may be envisioned as a novel way to prevent the deleterious effects of ROS-mediated diseases in general and of Ang II on the heart in particular.

## 1.2 Signaling of the E-cadherin cell-cell adhesion protein

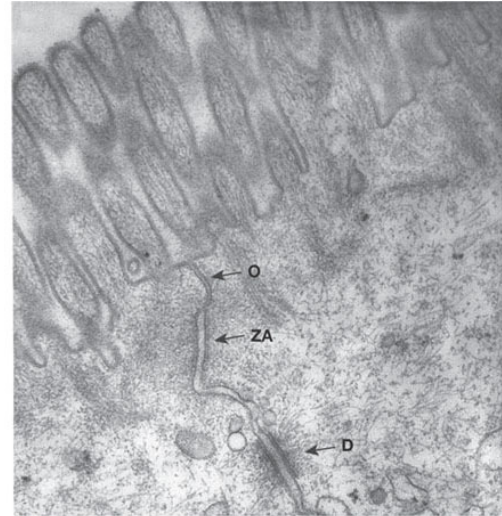
The cadherins constitute a major class of adhesion molecules that support calcium-dependent, homophilic cell-cell adhesion in all solid tissues of the body. They mediate cell-cell recognition events, bring about morphological transitions that underlie tissue formation, and maintain tissue architecture in the adult organism. The next paragraph will give a brief introduction of E-cadherin-dependent cell-cell adhesion with major emphasis on its tumor suppressing function and its signaling capacities.

### 1.2.1 E-cadherin-dependent cell-cell adhesion

#### 1.2.1.1 E-cadherin: a member of the classical cadherins

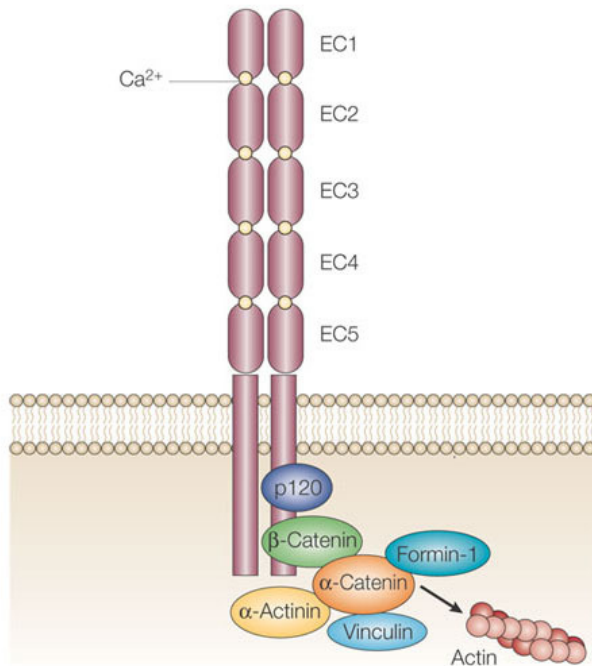
Cadherins represent a large superfamily which includes classical cadherins, desmosomal cadherins, atypical cadherins, proto-cadherins and cadherin-related signaling molecules (Gumbiner, 2005). E-cadherin is a prototype family member and belongs to the classical cadherins. Classical cadherins were originally named for the tissue in which they are most prominently expressed. Later, it became clear that most cadherins can be expressed in many different tissues. E-cadherin (epithelial cadherin) is expressed primarily in epithelial cells and is associated with the zonula adherens (which is also known as adherens junctions) of the epithelial junctional complex (Fig. 1.2.1.1-1). Adherens

junctions represents a specialized form of cadherin-based adhesive contacts which helps cells to form a tight, polarized cell layer that can perform barrier and transport functions (Gumbiner, 2005).



**Figure 1.2.1.1-1: Epithelial junctional complex.** Adhesion between vertebrate cells is generally mediated by three types of adhesion junction: adherens junction (zonula adherens), tight junction (zonula occludens), and desmosomes. Electron micrograph of an epithelial junctional complex containing zonula adherens (ZA), zonula occludens (O), and desmosome (D). The ZA junction completely encircles the apex of the epithelial cell, but only a section through the junction is shown. The membranes of the two cells align tightly at the junction, with an extracellular gap of 250Å. The cytoplasmic surface of the junction appears as a dense plaque, presumably made up of cytoskeletal proteins, which associates with actin filament (taken from (Gumbiner, 2005)).

Classical cadherins are single-pass transmembrane proteins. They contain five cadherin domains on their extracellular part which confer specific adhesive binding, and homophilic protein-protein interactions



**Figure 1.2.1.1-2: The classical cadherin-catenin complex.** Cadherin is a parallel, or *cis*, homodimer. The extracellular region of classical cadherins consists of five cadherin-type repeats (extracellular cadherin domains) that are bound together by  $\text{Ca}^{2+}$  ions (yellow circles) to form stiff, rod-like proteins. The core universal-catenin complex consists of p120-catenin, bound to the juxtamembrane region, and  $\beta$ -catenin, bound to the distal region, which in turn binds  $\alpha$ -catenin. In a less well understood way,  $\alpha$ -catenin binds to actin and actin-binding proteins, such as vinculin,  $\alpha$ -actinin, or formin-1 (taken from (Gumbiner, 2005)).

between two cadherin molecules on two cells. The exact structure of the homophilic bond is still a matter of debate (Gumbiner, 2005), but an intriguing possibility is that some of the existing models represent different conformational states that are important for the regulation of adhesion. The presence of a conserved cytoplasmic tail that associates with

cytoplasmic proteins, the catenins, is a second characteristic which distinguishes classical cadherins from other members of the cadherin superfamily (Fig. 1.2.1.1-2) (Takeichi, 1995).  $\alpha$ -catenin interacts, through  $\beta$ -catenin, with the distal part of the cadherin cytoplasmic domain.  $\gamma$ -catenin (also known as plakoglobin) can bind to the same site as  $\beta$ -catenin in a mutually exclusive way, whereas another catenin, p120-catenin, interacts with a more proximal region of the cytoplasmic domain.

### 1.2.1.2 Function of catenins in the E-cadherin adhesion complex

The main function of catenins is the conversion of the specific homophilic binding capacity of the E-cadherin extracellular domain into a stable cell-cell adhesion. Although the E-cadherin extracellular domain alone possesses homophilic binding properties, stable cell adhesion requires the cadherin cytoplasmic tail and associated proteins (Yap et al., 1997).

$\alpha$ -catenin can mediate physical links between cadherin and the actin cytoskeleton, either by directly binding actin filaments or indirectly through other actin-binding proteins such as vinculin and  $\alpha$ -actinin (Fig. 1.2.1.2A). Besides linking cadherins to the actin cytoskeleton, catenins are believed to play additional roles.  $\beta$ -catenin is a well known signaling molecule in the Wnt pathway (see below), and catenins can interact with other signaling molecules, such as GTPases (Goodwin et al., 2003), PI3K (Woodfield et al., 2001), and formin-1 (known to nucleate actin polymerisation) (Kobielak et al., 2004), to influence the state of the actin cytoskeleton (see below) (Fig. 1.2.1.2B).

The core function of p120-catenin is to regulate cadherin turnover (Reynolds and

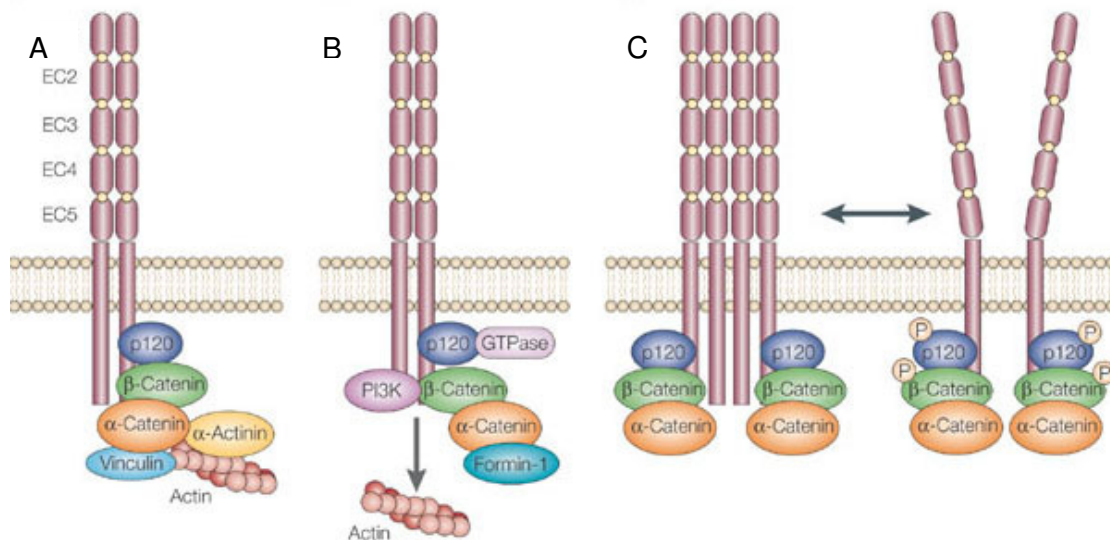
Roczniak-Ferguson, 2004). Loss of p120-catenin leads to significantly reduced levels of E-cadherin in epithelial cells (Davis et al., 2003). Thus, p120-catenin directly influences adhesive strength by controlling the amount of E-cadherin available at the cell surface for adhesion.

Furthermore, the adhesive strength of cadherins is changed by posttranslational modifications of p120-catenin and  $\beta$ -catenin. Although poorly understood, tyrosine phosphorylation of catenins is believed to regulate the conformation or organization of cadherins. It is thought that phosphorylation of catenins could lead to a disruption of dimerization and reduced clustering of the cadherin molecules at the surface, resulting in

an inactive, or less adhesive, conformation (Fig. 1.2.1.2C).

### 1.2.1.3 Function of the E-cadherin-catenin complex

The E-cadherin-catenin complex is essential for the formation of epithelia in the embryo, and maintenance of epithelial structure in the adult. It carries out different functions, including cell-cell adhesion, cytoskeletal anchoring, and signaling. The expression of different types of cadherins mediates selective cell recognition events that are responsible for the sorting of different groups of cells in developing tissues, and the formation of selective connections between neurons in the developing nervous



**Figure 1.2.1.2: Function of catenin proteins in the E-cadherin-catenin complex.** There are three ways in which catenins contribute to the cadherin function. **(A)**  $\alpha$ -catenin provides a direct physical link to the actin cytoskeleton through interaction with E-cadherin-bound  $\beta$ -catenin and actin or actin-binding proteins such as vinculin and  $\alpha$ -actinin. **(B)** Catenins bind to or influence signaling molecules (GTPases, formin-1, PI3K) known to control the actin cytoskeleton. **(C)** Phosphorylation of catenins might control the adhesive strength of the cadherin-catenin complex. Depicted is a hypothetical example where phosphorylation of catenins could lead to a disruption of dimerization and reduced clustering of cadherin molecules at the cell surface, resulting in an inactive or less adhesive conformation.  $\text{Ca}^{2+}$  ions are indicated by yellow circles. EC: extracellular cadherin domain (taken from (Gumbiner, 2005)).

system (Gumbiner, 2005). In cell culture, a mixed population of cells expressing different cadherins become sorted by adhering only to those cells expressing the same cadherin (Yap et al., 1997). During development, segregation of cells into distinct tissues is accompanied by changes in the complement of cadherins expressed by the cells. The specificity of homophilic binding is therefore a fundamental mechanism by which cadherins influence the organization of various cell types into tissue (Yap et al., 1997). However, different cadherins can be promiscuous with regards to their adhesive binding properties, with evidence for heterophilic adhesion between different classical cadherins. The level of cadherin expression, and presumably therefore the overall strength of adhesion, has also been found to strongly influence cell-sorting behavior, independently of the type of cadherin expressed (Gumbiner, 2005).

The importance of E-cadherin-mediated cell adhesion is also highlighted by the fact that its disturbance is causally involved in cancer development.

### 1.2.2 E-cadherin as a tumor suppressor

The majority of human cancers (ca. 80-90%) originate from epithelial cells. In most, if not all, of these epithelial-derived cancers, E-cadherin-mediated cell-cell adhesion is lost, concomitant with the transition from benign, non-invasive tumor to malignant, invasive tumor. Although E-cadherin expression is maintained in most differentiated tumors, including carcinomas of the skin, head and neck, breast, lung, liver, colon, and prostate, there seems to be an inverse correlation between E-cadherin levels and cancer grade (Birchmeier and Behrens,

1994; Hirohashi, 1998). This observation has prompted an examination of the functional role of E-cadherin in tumor progression. Behrens *et al.* (Behrens et al., 1989) showed that epithelial cells acquire invasive properties when intercellular adhesion is specifically inhibited by the addition of E-cadherin function-blocking antibodies; the separated cells then invade collagen gels and embryonic heart tissue. Subsequently, several groups have demonstrated that re-establishing the functional cadherin complex by forced expression of E-cadherin results in a reversion of an invasive, mesenchymal phenotype to a benign, epithelial phenotype of cultured tumor cells (Birchmeier and Behrens, 1994; Navarro et al., 1991; Vleminckx et al., 1991). Based on these data, it has been proposed that the loss of E-cadherin-mediated cell-cell adhesion is a prerequisite for tumor cell invasion and metastasis formation. The *in vivo* proof that loss of E-cadherin is not a consequence of de-differentiation, but rather the cause of tumor progression, was made by Christofori and colleagues (Perl et al., 1998). Intercrossing *RipTag2* mice, which provide a model of pancreatic carcinogenesis, with transgenic mice that maintain E-cadherin expression in  $\beta$ -cell-derived tumor cells resulted in the arrest of tumor development at the adenoma stage, whereas expression of a dominant-negative form of E-cadherin induced early invasion and metastasis. Very recently, a second study has demonstrated causal evidence for the involvement of E-cadherin in tumor progression. A group from the Netherlands introduced a conditional loss-of-function mutation in the E-cadherin gene into mice that carry *p53* mutations. Although tissue-specific inactivation of E-cadherin alone did not result in tumor formation, the combined inactivation



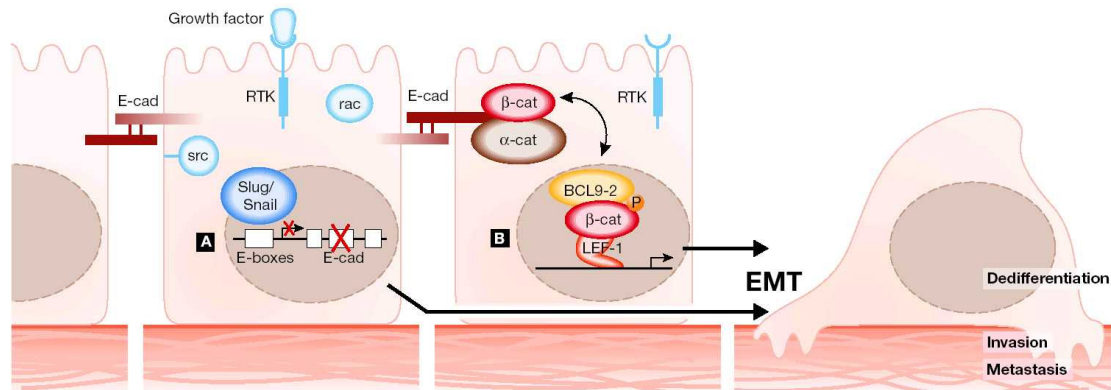
of E-cadherin and p53 led to the accelerated development of mammary gland and skin tumors. Moreover, loss of E-cadherin induced a phenotypic change from non-invasive to highly invasive mammary gland tumors, and a conversion from ductal to lobular carcinomas (Birchmeier, 2005). These results show that the loss of E-cadherin-mediated cell-cell invasion is one rate-limiting step in the progression from adenoma to carcinoma and subsequent formation of tumor metastases.

Downregulation of E-cadherin is often part of a process called epithelial-to-mesenchymal transition (EMT), which is characterized by the loss-of-expression of epithelial genes and the gain-of-expression of mesenchymal genes (Thiery, 2002). EMT is a crucial event during tumor metastasis but also occurs in normal embryonic development, for example during gastrulation (Fig. 1.2.2). Activation of RTK [fibroblast growth factor receptor (FGFR), EGFR family, transforming growth factor- $\beta$  (TGF- $\beta$ ) receptor, insulin-like growth factor receptor (IGFR), hepatocyte growth factor receptor (HGFR)] signaling is able to induce EMT via stimulation of PI3K, Src, Ras and Rac. Signaling downstream of EGFR, c-Met and FGFR, as well as Src, results in tyrosine phosphorylation of E-cadherin,  $\beta$ -catenin and p120-catenin, leading to a disassembly of the cadherin-catenin complex, disruption of cadherin-mediated adhesion and cell scattering. Tyrosine phosphorylation-mediated ubiquitination and subsequent proteasomal degradation of E-cadherin or increased endocytosis of E-cadherin seem to be mechanisms underlying this observed disassembly (Fujita et al., 2002; Kamei et al., 1999). Moreover, induction of expression of

transcription factors, such as Snail and Slug, has been observed downstream of RTK signaling (Thiery, 2002). Snail, Slug, SIP1, and E12/47, as well as Twist, are factors which repress transcription from the *E-cadherin* promoter via the E-boxes (Cavallaro and Christofori, 2004; Yang et al., 2004).

$\beta$ -catenin is also actively involved in EMT (Fig. 1.2.2) and its role as a signaling molecule will be discussed later.

In addition to EMT, which is a rather organized process leading to downregulation of E-cadherin expression, various other mechanisms are involved in the disruption of cell-cell adhesion during tumor progression. A variety of genetic mechanisms, such as deletion or mutational inactivation of the gene, or gene mutations which result in the expression of a non-functional protein, cause loss of E-cadherin expression or function, especially in diffuse gastric cancer (Birchmeier and Behrens, 1994; Bracke et al., 1996; Strathdee, 2002). Silencing of the *E-cadherin* gene by hypermethylation of promoter regions occurs frequently in carcinoma cell lines, in thyroid carcinomas, and in several other cancer types (Di Croce and Pelicci, 2003; Hirohashi, 1998). More recently, proteolytic degradation of E-cadherin by matrix-metallo proteases (MMPs) has been described as a mechanism by which cell-cell adhesion can be disrupted. Cleavage of E-cadherin results in not only the disruption of cell-cell adhesion, but also the production of a soluble 80-kDa E-cadherin fragment that itself disrupts cell-cell adhesion in a dominant-interfering manner, thereby promoting tumor progression (Noe et al., 2001; Wheelock et al., 1987).



**Figure 1.2.2: Epithelial-mesenchymal transition (EMT).** Epithelial cells lose the expression of epithelial-specific genes, such as E-cadherin, and acquire the expression of mesenchymal genes (vimentin, collagens, integrins). EMT causes cells to lose apical-basal polarity (shown on the left) and gain a fibroblast-like morphology, high motility and invasive properties (shown on the right). **(A)** Transcription factors (such as Snail and Slug) have been identified that control the expression of E-cadherin by binding directly to E-boxes in the gene promoter. Other factors, such as growth factors and their receptors, the tyrosine kinase src, and cytoplasmic G-proteins (such as rac) can also promote EMT indirectly. **(B)**  $\beta$ -catenin was found to exert a dual role as an essential cytoplasmic-interaction partner of cadherins, which is essential for cell-cell adhesion, and as a nuclear partner of the T-cell factor (TCF)/lymphocyte-enhancer factor (LEF) family of transcription factors that regulate genes of the canonical Wnt signaling pathway. The switch of  $\beta$ -catenin from its action in cell adhesion to transcriptional control in the nucleus is controlled by binding to BCL9-2, which is the homologue of a human B-cell oncogene product, and is promoted by tyrosine phosphorylation of  $\beta$ -catenin (taken from (Birchmeier, 2005)).

As already mentioned above, appropriate cell-cell adhesion requires the cadherin-catenin complex as a whole. Therefore, changes in the expression of catenins, for example mutations in  $\alpha$ -catenin or expression of truncated  $\alpha/\beta$ -catenin, impair E-cadherin-mediated cell adhesion and are often associated with malignant transformation (Hajra and Fearon, 2002; Hirohashi and Kanai, 2003). Recently it has been shown that knockdown of p120-catenin results in the destruction of the entire cadherin complex (Reynolds and Rocznik-Ferguson, 2004). Together with evidence of frequent p120-catenin loss in cancer, these observations suggest that p120-catenin downregulation itself may be an initiating event

in a subset of E-cadherin-deficient tumors. However, direct evidence is lacking and it remains to be determined whether this would represent a general process in tumor progression.

Proper E-cadherin function can also be overruled or replaced by the expression of mesenchymal cadherins, such as N-cadherin, which has been shown to promote cell motility and migration. It becomes more and more evident that this “cadherin switch” is involved during the transition from a benign to an invasive tumor phenotype (Christofori, 2003).

Taken together, loss of E-cadherin-mediated cell-adhesion strongly contributes to tumor progression, but it is unlikely that loss of E-



cadherin by itself can account for the metastatic phenotype, because loss of adhesiveness does not necessarily cause cells to become motile and/or invasive; additional events are required.

### 1.2.3 E-cadherin-mediated signaling

An increasing body of evidence suggests that cadherins act at the cellular level as adhesion-activated cell signaling receptors (Cavallaro and Christofori, 2004; Wheelock and Johnson, 2003). Although signals that are elicited by the formation of E-cadherin-dependent cell-cell adhesion have been extensively studied, signals that are induced by the loss of E-cadherin function, for example during cancer progression, are only just being elucidated.

Several studies have reported that establishment of E-cadherin-mediated contact influences the activity of Rho-family GTPases; with Rac and CDC42 being activated and Rho being inactivated. The mechanisms underlying this activation or inactivation vary depending on the model system used. One connection between cadherins and Rho GTPases is through p120-catenin. It has been shown that p120-catenin activates Rac1 and CDC42, perhaps by activating Vav2, which is a guanine exchange factor for these GTPases (Fig. 1.2.3) (Grosheva et al., 2001; Noren et al., 2001). Reynolds and colleagues showed that cytosolic p120-catenin inhibits RhoA activity by acting as guanine nucleotide dissociation inhibitor (Anastasiadis et al., 2000; Noren et al., 2000). It is worth noting that only cytosolic p120-catenin is able to modulate GTPase activity; this function is abolished when p120-catenin participates in the E-cadherin adhesion complex. Rho activity decreases as cells

become confluent, but as cytosolic p120-catenin becomes sequestered by the E-cadherin adhesion complex it cannot account for this decrease in Rho activity. Therefore, other mechanisms downstream of E-cadherin-mediated adhesion decrease Rho activity. Noren et al. (Noren et al., 2003) reported that E-cadherin engagement in cell-cell adhesion suppresses Rho activity by inducing phosphorylation and activation of p190RhoGAP, probably through Src-family kinases. In other systems, E-cadherin was found to communicate with Rho GTPases via PI3K signaling (Fig. 1.2.3). PI3K is an upstream kinase of Rac and has previously been found to interact with E-cadherin (Pece et al., 1999; Woodfield et al., 2001). Yap and colleagues (Kovacs et al., 2002) showed that PI3K co-localized with E-cadherin at the leading edge of cadherin-based lamellipodia, and was necessary for full and sustained activation of Rac. In contrast, another group reported that Rac activation induced by E-cadherin ligation was independent of PI3K activity, but dependent on EGFR signaling (see below) (Betson et al., 2002). Whatever the mechanisms are, E-cadherin-mediated contacts influence the activity of Rho-family GTPases, which are believed to regulate dynamic organization of the actin cytoskeleton and the activity of the cadherin/catenin apparatus to modulate stabilization of the adhesive contact (Yap et al., 1997).

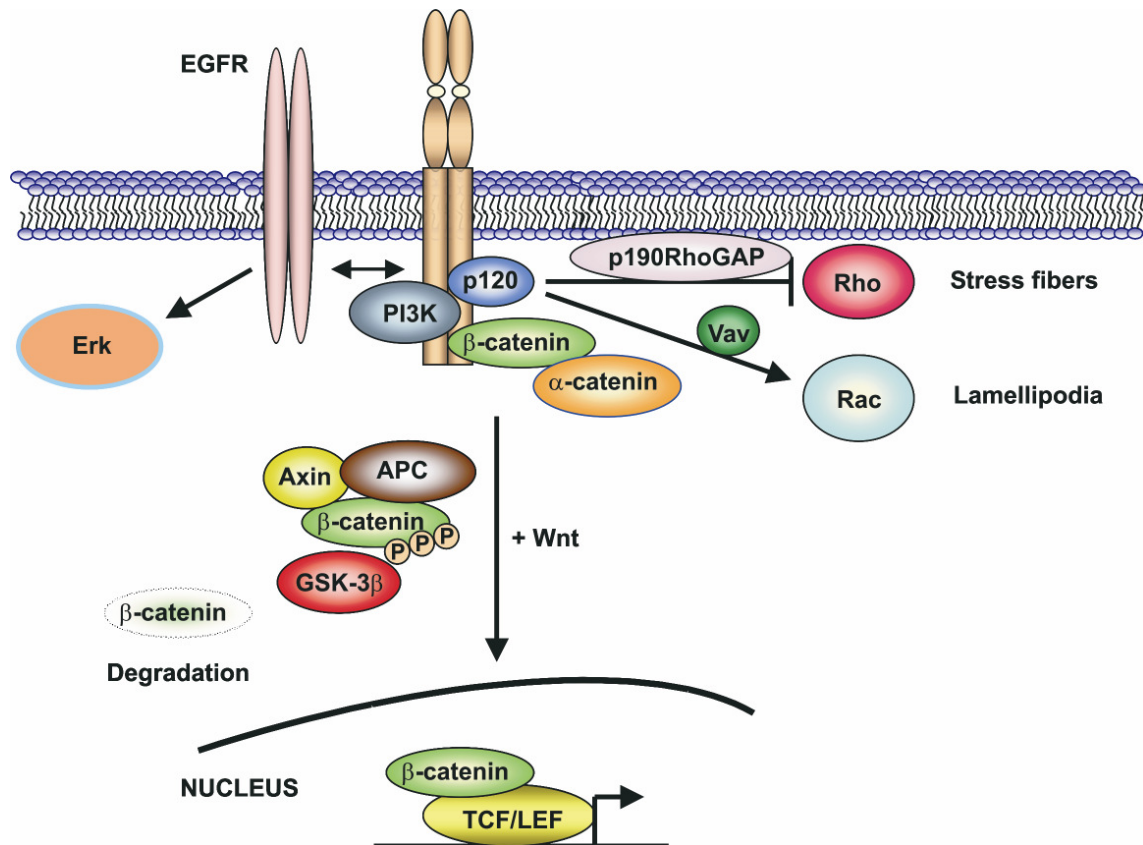
Several studies have suggested functional interdependence of cadherins and RTK with respect to their signaling capacities. It has been demonstrated that initiation of *de novo* E-cadherin-mediated adhesive contacts can induce ligand-independent activation of the EGFR and subsequent activation of Erk (Munshi et al., 2002; Pece and Gutkind, 2000).

In contrast, it has been shown that the E-cadherin adhesive complex can be linked to EGFR via  $\beta$ -catenin (Hoschuetzky et al., 1994) or via the extracellular domain of E-cadherin, and negatively regulate receptor tyrosine kinase signaling in an adhesion-dependent manner. Interaction of cadherins with respective RTK has been observed in different systems (VEGFR with VE-cadherin, and FGFR with N-cadherin) (Carmeliet et al., 1999; Cavallaro et al., 2001).

It is worth noting that  $\beta$ -catenin, besides being a major component of the E-cadherin adhesion complex, is also part of the Wnt-mediated signaling pathway. In the absence of Wnt signaling, cytosolic  $\beta$ -catenin is degraded through a pathway that is dependent on adenomatous polyposis coli protein (APC). However, upon stimulation of the Wnt pathway this degradation is suppressed, resulting in the accumulation of cytoplasmic  $\beta$ -catenin (Wheelock and Johnson, 2003). Subsequently, it translocates into the nucleus and acts as a coactivator of the T-cell factor (TCF)/lymphocyte-enhancer factor (LEF) transcription factors (Fig. 1.2.3). Given that binding to  $\beta$ -catenin precludes its participation in Wnt signaling, E-cadherin could potentially regulate Wnt signaling by sequestering  $\beta$ -catenin from TCF/LEF transcription factors. Gumbiner and colleagues (Gottardi and Gumbiner, 2004) reported that the participation of  $\beta$ -catenin in adhesion and Wnt signaling is dictated by the presence of distinct molecular forms of  $\beta$ -catenin that have different binding properties. More recently it has been shown that this switch can be regulated by the binding of  $\beta$ -catenin to BCL9-2 (the homolog of the human B-cell oncogene product BCL-9).  $\beta$ -catenin/BCL9-2 binding can be promoted by tyrosine phosphorylation of  $\beta$ -catenin, and

competes with  $\alpha$ -catenin binding and cell adhesion (Brembeck et al., 2004) (Fig. 1.2.2).

Although some groups have shown that overexpression of E-cadherin fragments able to bind  $\beta$ -catenin can repress TCF/LEF transcriptional activity in 293T cells (Simcha et al., 2001) or SW480 cells (Gottardi and Gumbiner, 2004), another group was unable to find any dependence of TCF/LEF-mediated transcriptional activity on E-cadherin expression in human breast cancer cells (van de Wetering et al., 2001). The effects of E-cadherin on Wnt signaling appear, therefore, to be cell-context-dependent.



**Figure 1.2.3: Signaling by the E-cadherin complex.** Initiation of de novo cell-cell adhesion activates Rac and CDC42, and inhibits Rho. Soluble p120-catenin and PI3K are most likely mediating these effects via activation of the respective guanine exchange factor (VAV) or GTPase-activating protein (p190RhoGAP). Establishment of E-cadherin-mediated contacts can also induce ligand-independent activation of the EGFR and, subsequently, activation of Erk and PI3K signaling. Cytosolic  $\beta$ -catenin is normally degraded through the adenomatous poliposis coli (APC) complex. However, WNT signaling inhibits the APC complex, allowing  $\beta$ -catenin to enter the nucleus and coactivate TCF/LEF transcription factors. By sequestering  $\beta$ -catenin from participation in the WNT signaling, the E-cadherin adhesion complex might also modulate WNT-induced transcription.

### 1.3 RNA interference: a new and powerful tool in molecular biology

RNAi is a general term for sequence-specific gene repression induced by double-stranded RNAs (dsRNAs) that was initially discovered in plants. It was later observed in the animal model organism *Caenorhabditis elegans* that dsRNA triggered sequence-specific mRNA cleavage (Fire et al., 1998). It soon turned out that RNAi is not restricted to nematode and can be induced in *Drosophila melanogaster* (Kennerdell and Carthew, 1998), *Trypanosoma* (Ngo et al., 1998), and vertebrates (Elbashir et al., 2001a; Yang et al., 2001).

During RNAi, long dsRNA molecules are processed into 19- to 23-nt RNAs known as small-interfering RNAs (siRNAs) that serve as guides for enzymatic cleavage of complementary RNAs (Elbashir et al., 2001b; Parrish et al., 2000; Zamore et al., 2000). In *Drosophila* and *C. elegans*, siRNAs can function as primers for an RNA-dependent RNA polymerase that synthesizes additional dsRNA, which in turn is processed into siRNAs, amplifying the effects of the original siRNAs (Lipardi et al., 2001; Sijen et al., 2001).

In mammalian cells, the experimental use of RNAi with dsRNA has not been successful in most cell types because of non-specific responses elicited by dsRNA molecules longer than about 30 nt (Robertson and Mathews, 1996). Tuschl and coworkers (Elbashir et al., 2001a) discovered that transfection of synthetic 21-nt siRNA duplexes into mammalian cells effectively inhibits endogenous genes in a sequence-specific manner. These siRNA duplexes are too short

to trigger the non-specific dsRNA responses, but they still cause destruction of complementary RNA sequences (Gitlin et al., 2002).

More recently, a large number of endogenous microRNA (miRNAs) was discovered. miRNAs are a specific class of small RNAs that are encoded in gene-like elements organized in a characteristic inverted repeat (Grishok et al., 2001; Reinhart et al., 2000). Because the active forms of miRNAs and siRNAs are sometimes biochemically or functionally indistinguishable, they are classified based on their origins (Fig. 1.3). siRNAs are derived from long dsRNAs in the cytoplasm, whereas miRNA genes are transcribed by RNA polymerase II to generate long primary transcripts (pri-miRNAs) (Cai et al., 2004; Lee et al., 2004). In the nucleus, pri-miRNAs are trimmed to release hairpin intermediates (pre-miRNAs) (Lee et al., 2002) by the RNase III type enzyme Drosha (Lee et al., 2003). pre-miRNAs then get exported to the cytoplasm (Bohnsack et al., 2004), where they are processed in a similar way to dsRNAs, the precursors of siRNAs. pre-miRNAs and dsRNAs are processed by Dicer, the cytoplasmic RNase III type protein (Bohnsack et al., 2004; Hutvagner et al., 2001; Ketting et al., 2001) and cleaved into the short-lived miRNA/siRNA duplexes, whose one strand is degraded by an unknown nuclease while the other strand remains as a mature miRNA/siRNA (Khvorova et al., 2003; Schwarz et al., 2003). The released miRNAs/siRNAs are incorporated into silencing complexes. Although it is difficult to assign a distinct functional label, an siRNA-containing complex is commonly referred to as RNA-induced silencing complex (RISC), whereas an miRNA-

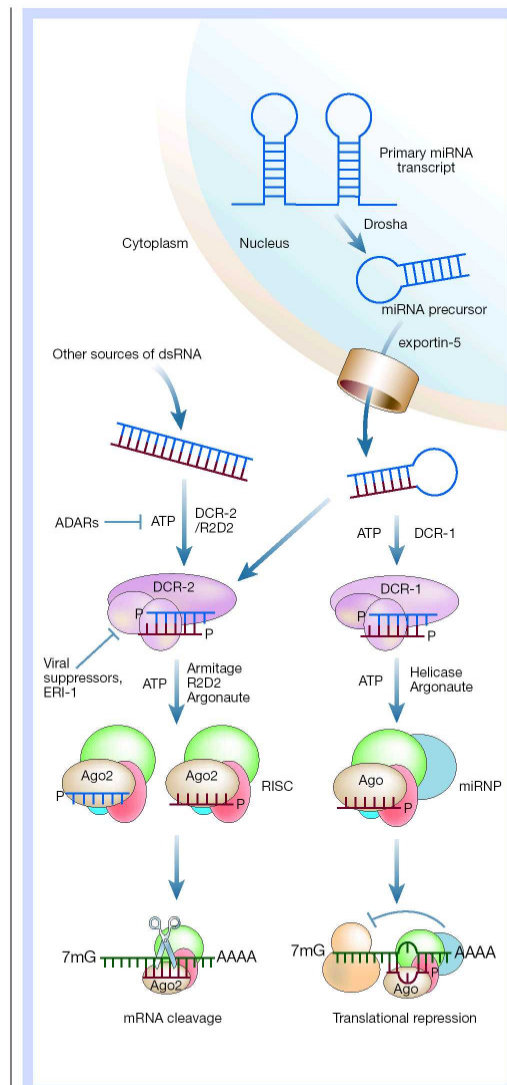
containing effector complex is referred to as a micro ribonucleoprotein particle (miRNP) (Meister and Tuschl, 2004). Every RISC or miRNP contains a particular subset of Argonaute proteins that exert sequence-specific gene repression by inducing cleavage ('slicing') or, as in the case of several miRNPs, by eliciting a block to translation (Meister and Tuschl, 2004).

miRNAs are often only temporarily expressed and seem to play a role in developmental processes. In mammals, miR-181 is involved in the control of hematopoiesis through as yet unknown target(s) (Chen et al.,

2004). Mouse miR-196 miRNAs repress the expression of the *hoxb8* gene, a transcription factor important during vertebrate developmental regulation (Yekta et al., 2004). Several reports have also shown that altered expression of specific miRNA genes contributes to the initiation and progression of cancer (Croce and Calin, 2005; Gregory and Shiekhattar, 2005; McManus, 2003). All these findings prove that miRNAs play important regulatory roles in animals by targeting the messages of protein-coding genes for translational repression or degradation.

**Figure 1.3: Model of small-RNA-guided post transcriptional regulation of gene expression.**

Primary miRNA transcripts are processed to miRNA precursors in the nucleus by the RNase-III-like enzyme Drosha. The miRNA precursor is subsequently exported to the cytoplasm by means of the export receptor exportin-5. The miRNA precursor is further processed by Dicer to siRNA-duplex-like intermediates. The duplex is unwound while assembling into miRNP/RISC. Mature miRNAs bind to Ago proteins, which mediate translational repression or cleavage of target mRNAs. Other sources of long dsRNA in the cytoplasm of a cell are viral RNAs, artificially introduced dsRNA, and genomic sense and antisense transcripts. Like miRNA precursors, long dsRNA is processed by the RNase III enzyme Dicer into 21-23 nucleotide dsRNA intermediates. Assisted by the RNA helicase Armitage and R2D2, the single-stranded siRNA-containing RISC is formed. The stability of the dsRNA, and its recognition by Dicer, can be regulated by specific ADARs (deaminase) and the exonuclease ERI-1. DCR: Dicer-like protein, R2D2: dsRNA binding protein (taken from (Meister and Tuschl, 2004)).



In contrast, siRNAs are produced from dsRNAs that are synthesized from viruses, endogenously activated transposons, or repetitive sequences introduced by genetic engineering. Thus, siRNAs have been proposed to function in: (i) antiviral defense, (ii) silencing mRNAs that are overproduced or translationally aborted, and (iii) guarding the genome from disruption by transposons (Hannon, 2002; Mello and Conte, 2004; Tabara et al., 1999). In experimental research, siRNAs can efficiently and rapidly downregulate the level of an endogenous protein in mammalian cells. The use of siRNA therefore complements overexpression studies in tissue culture by providing a powerful tool to investigate loss-of-function of a given protein.

## 1.4 Research objectives

Several reports have shown that Shc adaptor proteins are involved in MAPK activation induced by several growth factors. However, the contribution of each isoform in mediating this process is still not known. Moreover, are there other signaling pathways in which Shc might play a role, despite growth factor-induced signaling? The largest isoform, p66<sup>Shc</sup>, does not seem to be involved in MAPK activation, but whether it acts in a dominant negative manner or in a neutral way is still a matter of debate. Gene targeting of p66<sup>Shc</sup> has revealed that this isoform is implicated in the regulation of lifespan and the response to oxidative stress. The absence of p66<sup>Shc</sup> confers resistance to oxidative stress on mouse embryo fibroblasts, endothelial cells, and T-cells. Still, it is unclear whether this is a general effect or restricted to certain cell types and what the underlying mechanisms are.

This thesis aims to address some of these questions. To investigate isoform-specific functions of Shc proteins, we first generated a system to specifically knockdown single Shc isoforms using siRNA. This method was developed further to the so-called “knockdown-in system”, where we achieved an isoform-specific expression of Shc proteins. These data will be discussed in greater detail in the first part of the following chapter.

Secondly, we used this method to explore how necessary Shc proteins are in EGF-induced signaling. In a quest for growth factor-independent pathways in which Shc proteins might be involved, we investigated signaling downstream of the cell adhesion molecule, E-cadherin. These results are described in sections 2.3-2.5. The last part of the following

chapter represents data on the role of p66<sup>Shc</sup> in regulating cell viability upon stress response in epithelial cells.

## 2. RESULTS

### 2.1 RESEARCH COMMUNICATION

#### Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA

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Many eukaryotic genes are expressed as multiple isoforms through the differential utilization of transcription/translation initiation sites or alternative splicing. The conventional approach for studying individual isoforms in a clean background (i.e. without the influence of other isoforms) has been to express them in cells or whole organisms in which the target gene has been deleted; this is time-consuming. Recently an efficient post-transcriptional gene-silencing method has been reported that employs a small interfering double-stranded RNA (siRNA). On the basis of this method we report a rapid alternative approach for isoform-specific gene expression. We show how the adaptor protein ShcA can be suppressed and expressed in an isoform-specific manner in a human cell line. ShcA exists in three isoforms, namely p66, p52 and p46, which differ only in their N-terminal regions and are derived from two

different transcripts, namely p66 and p52/p46 mRNAs. An siRNA with a sequence shared by the two transcripts suppressed all of them. However, another siRNA whose sequence was present only in p66 mRNA suppressed only the p66 isoform, suggesting that the siRNA signal did not propagate to other regions of the target mRNA. The expression of individual isoforms was achieved by first down-regulating all isoforms by the common siRNA and then transfecting with an expression vector for each isoform that harboured silent mutations at the site corresponding to the siRNA. This allowed functional analysis of individual ShcA isoforms and may be more generally applicable for studying genes encoding multiple proteins.

Key words: post-transcriptional gene silencing, RNAi, transient knockdown-in.

### INTRODUCTION

In eukaryotes, many genes encode multiple isoforms by way of differential transcription/translation initiation or alternative splicing, thus giving rise to related proteins with biochemically as well as biologically distinct features [1]. Although, in many cases, multiple isoforms are expressed in the same cell at the same time, the expression level and pattern of each isoform may vary with the cell type and its stage of development, making the study of each isoform confusing and difficult. Investigation of the function of individual isoforms ideally requires conditions where only one isoform is expressed or eliminated. While several protocols for tissue-specific expression or elimination of the gene of interest have been developed [2–4], reports of isoform-specific gene inactivation [5] or expression in a clean background are limited. Such protocols are usually lengthy, often requiring several months to establish the desired conditions. During this time, cells or organisms may adapt to the new conditions [6] and caution is required when interpreting the results.

The signalling adaptor/scaffold protein ShcA is a member of the Shc family, which consists of three genes, *ShcA*, *ShcB/Sli/Sck* and *ShcC/N-Shc/Rai* [7]. ShcA is ubiquitously expressed, whereas ShcB and

ShcC are expressed specifically in the brain [8]. ShcA is recruited to, and phosphorylated by, activated receptor tyrosine kinases and, in turn, recruits the growth-factor-receptor-bound protein 2 (Grb2)–Son-of-sevenless ('Sos') complex via the Src homology 2 (SH2) domain of Grb2, thus relaying growth-factor-induced signals to the Ras/extracellular-signal-regulated protein kinase ('ERK') signalling pathway [9]. It is also involved in growth-factor-mediated activation of c-Jun N-terminal kinase ('JNK') and protein kinase B, but the precise mechanism is unknown [9]. There are three isoforms of ShcA, namely p66ShcA, p52ShcA and p46ShcA, derived from a single gene through differential usage of transcription initiation sites and translation start sites, which differ only in the N-terminal regions [7]. While all three isoforms contain three tyrosine residues in the phosphotyrosine-binding (PTB) domain that are phosphorylated by activated receptor tyrosine kinases, they differ in the pattern of serine/threonine phosphorylation induced by growth factors and tumour phorbol esters. This suggests a different cellular function for each isoform ([5, 10]; A.F. El-Shemerly, A. Faisal and Y. Nagamine, results not shown). However, a systematic analysis of each isoform without the influence of other isoforms has not been reported.



Post-transcriptional gene silencing (PTGS) is a phenomenon originally reported in plants [11, 12], where introduction of the transgene causes silencing of the endogenous homologous gene and itself. The mechanism of PTGS involves enhanced mRNA degradation with double-stranded (ds)RNA as the trigger [13, 14]. A similar phenomenon (quelling) was observed in *Neurospora* [15]. In the Animal Kingdom, dsRNA-mediated gene silencing was first described in the nematode *Caenorhabditis elegans* [16] and was termed 'RNA interference' (RNAi). Subsequently, RNAi has been observed in a wide range of organisms, including flies, trypanosomes, *Hydra*, zebrafish (*Danio rerio*) and mice [13, 17]. The mechanism underlying RNAi has been partially elucidated, and a 21–23-nt-long dsRNA was found to be the intermediate/mediator of mRNA decay [18, 19]. Elbashir et al. [20] have shown recently that transfection of the 21-nt dsRNA, termed 'small interfering RNA' (siRNA), can trigger PTGS of both the co-transfected and the endogenous gene in cultured mammalian cells. In a cell-free system of dsRNA-mediated mRNA decay using *Drosophila* embryonal cell extracts, the mRNA was shown to be cleaved only within the region of identity with the dsRNA [18], suggesting that endonucleolytic cleavage induced by siRNA is very specific and that it is probably not propagated to other regions of mRNA. However, the interesting possibility of distinguishing closely related mRNAs by siRNA has not been addressed with mammalian cells.

In the present study, using transient transfection assays in HeLa cells, we established that the target of siRNA is restricted to mRNAs containing the identical sequence. This facilitated the isoform-specific knock-down of p66ShcA as well as isoform-specific expression of ShcA isoforms.

## EXPERIMENTAL

### Cells and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% (v/v) fetal-calf serum (AMIMED; BioConcept, Allschwil, Switzerland), 0.2mg/ml streptomycin and 50 units/ml penicillin at 37°C in a humidified 5% CO<sub>2</sub> incubator. A day before transfection with siRNA, cells were plated in six-well plates in medium without antibiotics at  $1.4 \times 10^5$  cells/well. The next morning, siRNAs were introduced into HeLa cells using the OLI GOFECT AMINE™ reagent (Life Technologies) according to the manufacturer's instructions, with 10 µl of 20 µM siRNA and 3 µl of transfection reagent/well. Transfection with expression vectors was carried out 2 days after the OLIG OFECTAMINE™ transfection using LIPOFE CTAMINE™ 2000 (Life Technologies).

### cDNA cloning of ShcA isoforms

The full-length mouse p46, p52 and p66ShcA cDNAs were isolated from NIH 3T3 cells by reverse transcriptase-PCR using the sense primers 5'-CGG AAT TCA TGG GAC CTG GGG TTT CCT ACT-3', 5'-CGG AAT TCA TGA ACA AGC TGA GTG GAG GCG-3' and 5'-CGG AAT TCA TGG ATC TTC TAC CCC CCA AGC CGA AGT A-3' respectively and the common antisense primer 5'-CGG AAT TCA CAC TTT CCG ATC CAC GGG TTG C-3'. Full-length ShcA cDNAs were initially cloned into pBluescriptII KS<sup>+</sup> and nucleotide sequences verified by the dideoxynucleotide-chain-termination procedure.

### Construction of expression vectors

The haemagglutinin (HA)-tagged expression vector pcDNA3HA was constructed by inserting the overlapping oligonucleotide pair 5'-CCC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT G-3' and 5'-AAT TCA GCG AAT TCT GGA ACA TCG TAT GGG TAA GCC ATG GTG GGG TAC-3' into the *KpnI*-*EcoRI* site of pcDNA3 (Invitrogen). To construct expression vectors for HA-tagged ShcA, p46HA, p52HA and p66HA, the full-length cDNAs of p46, p52 and p66 were inserted into the *EcoRI*-*EcoRV* site of pcDNA3HA. ShcA mutants in which potential internal initiation methionine codons were converted into leucine codons, thus expressing only the p66ShcA or 52ShcA forms, were created using the QuickChange site-directed mutagenesis kit (Stratagene). The overlapping oligonucleotide pair 5'-CTC CTC CAG GAC CTG AAC AAG CTG AGT G-3' and 5'-CAC TCA GCT TGT TCA GGT CCT GGA GGA G-3' was used to mutate Met<sup>65</sup> (start site for p52) to leucine in p66HA, resulting in p66HA-ml. Another overlapping oligonucleotide pair, 5'-CCA ACG ACA AAG TCC TGG GAC CCG GGG-3' and 5'-CCC CGG GTC CCA GGA CTT TGT CGT TGG-3', was used to mutate the initiation sites for p46 in both p66HA-ml and p52HA, resulting in p66HA-ML and p52HA-ML. Silent mutations were introduced into these vectors at the sites corresponding to h/m-shc siRNA as above using the overlapping oligonucleotide pair 5'-GGG GTT TCC TAC TTG GTG CG TAC ATG GGT TGT C-3' and 5'-CAC AAC CCA TGT AG C GGA CCA AGT AGG AAA CCC C-3' (mutated nucleotides underlined) to give p46HA-sm, p52HA-ML-sm and p66HA-ML-sm (h/m-shc means that the sequence of siRNA is common to both human and mouse shc sequences). Note that proteins expressed from these vectors are identical with the parent proteins.

### Oligoribonucleotides

The following 21-mer oligoribonucleotide pairs were used: h/m-shc siRNA from nt 677–697 (in the PTB domain), 5'-CUA CUU GGU UCG GUA

CAU GGG-3' and 5'-CAU GUA CCG AAC CAA GUA GGA-3'; and p66-shc siRNA from nt 236–256 [in the CH2 (collagen homology 2) domain], 5'-GAA UGA GUC UCU GUC AUC GUC-3' and 5'-CGA UGA CAG AGA CUC AUU CCG-3'. Entire sequences were derived from the sequence of human p66ShcA mRNA (accession number HSU7377) and its complement and each pair has a 3' overhang of 2nt on each side. Designed RNA oligonucleotides were 'blasted' against the GenBank®/EMBL database to ensure gene specificity. The RNA oligonucleotides were obtained from Microsynth (Balgach, Switzerland). Annealing was performed as described by Elbashir et al. [20]. The complementary two strands (each at 20µM) in 200µl of annealing buffer [100mM potassium acetate/30mM Hepes/KOH (pH 7.4)/2mM magnesium acetate] were heated for 1min at 90°C and then incubated for 1h at 37°C. An siRNA corresponding to nucleotides 753–773 of the firefly luciferase mRNA was used as a negative control.

### Western-blot analysis

At the times indicated, cells were lysed in a buffer containing 120mM NaCl, 50mM Tris, pH 8.0, and 1% Nonidet P40 plus Complete (Roche) protein inhibitor tablets. The whole-cell extracts (20 µg) were analysed by Western blotting using a polyclonal rabbit anti-Shc antibody (1:250; Transduction Laboratories), mouse monoclonal anti-Grb2 (1:1000; Transduction Laboratories) or a mouse monoclonal anti-b-tubulin antibody (1:1000; Sigma). We used anti-rabbit or anti-mouse horseradish peroxidase-linked antibodies from Amersham as secondary antibodies. An enhanced chemiluminescence (ECL®) detection method (Amersham) was employed, and the membrane was exposed to Kodak X-Omat LS film. Quantification of ShcA proteins was done using ImageQuant 5.0.

## RESULTS

### Efficient down-regulation of ShcA by siRNA

Three isoforms of ShcA are derived from a single gene through differential usage of transcriptional initiation sites (p66 versus p52/p46) and translational initiation sites (p52 versus p46) (Figure 1). The primary transcript of p52/p46 mRNA contains the entire sequence of p66 mRNA; however, the very-5' region of p66 mRNA is present in the first intron of p52/p46 mRNA, but is absent in the latter mRNA, having been spliced out. The p46 and p52 isoforms are derived from the same mRNA using different translation initiation sites. Target sites of two siRNAs used are shown in Figure 1 below the p66 ShcA mRNA. Note that the sequence of p66-shc siRNA is from a 5' region of human p66ShcA mRNA and is absent in p52/46 ShcA mRNA.

When HeLa cells were transfected with the h/m-shc siRNA, the levels of all three ShcA isoforms were strongly decreased 24h after transfection and reached less than 20% of the control after 48h and 4% after 60h (Figure 2A). The level of control protein (b-tubulin) was not affected under the conditions employed, and this was also the case for Grb2, a protein that specifically interacts with ShcA upon activation of growth-factor signalling [7]. Time-course analysis showed that the levels of all ShcA isoforms remained low until the fifth day after transfection, but started to increase thereafter (Figure 2B). The decrease in the three isoforms was uniform, suggesting that both p52/p46 and p66 ShcA mRNAs were equally targeted by the siRNA.

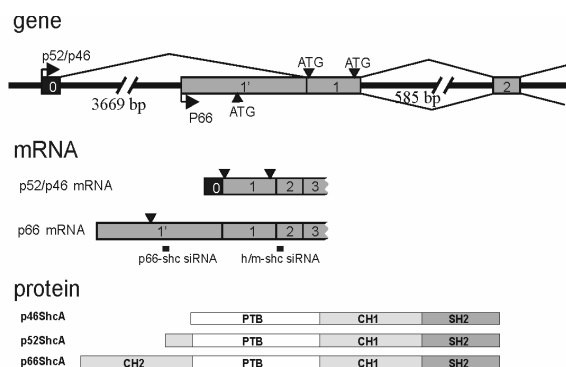
### Isoform-specific ShcA knockdown

When cells were transfected with p66-shc siRNA, only the p66ShcA isoform was decreased, with kinetics similar to that obtained with h/m-shc siRNA; the other two isoforms were not affected (Figure 3A and 3B). In another experiment, cells were challenged a second time with the same p66-shc siRNA 6 days after the initial transfection, when the level of p66ShcA was very low, but about to increase, and 10 days after the initial transfection, when the level of p66ShcA recovered substantially. As shown in Figure 3 (C), the level of p66ShcA remained low and decreased markedly again after transfection at days 6 and 10 respectively.

### Isoform-specific ShcA expression: transient knockdown-in (see below)

The target site of p66-shc siRNA is in the 5' region of p66 ShcA mRNA that is derived from the exon 1' and is absent in p52/p46 ShcA mRNA (see Figure 1). The results of the above experiments suggest that the effect of an siRNA is restricted to mRNAs containing a sequence identical with that of the siRNA used. Furthermore, there was no spreading effect of the siRNA signal, at least not towards the 3' of the target site in the mRNA. Otherwise, the p46 and p52 isoforms would also have been down-regulated by p66-shc siRNA. We exploited this specificity to establish conditions under which ShcA would be expressed in an isoform-specific manner, which we call 'transient knockdown-in'. We constructed expression vectors encoding mouse ShcA isoforms with or without silent mutations at the region corresponding to h/m-shc siRNA that left the protein sequences unchanged. Two point mutations were introduced into each expression vector so that it was not recognized by h/m-shc siRNA. Cells were first transfected with h/m-shc siRNA to knock-down all three isoforms and then 2 days later with an expression vector encoding each isoform of mouse ShcA. As shown in Figure 4, h/m-shc siRNA knocked down all three isoforms of endogenous

ShcA almost completely. Transfection of these cells with mutant expression vectors for individual isoforms resulted in the expression of only the corresponding isoforms. As expected, almost no protein was detected in cells transfected with wild-type expression vectors. Cells which were not transfected with h/m-shc siRNA expressed elevated levels of ShcA isoforms, irrespective of the presence or absence of mutations in the expression vectors.



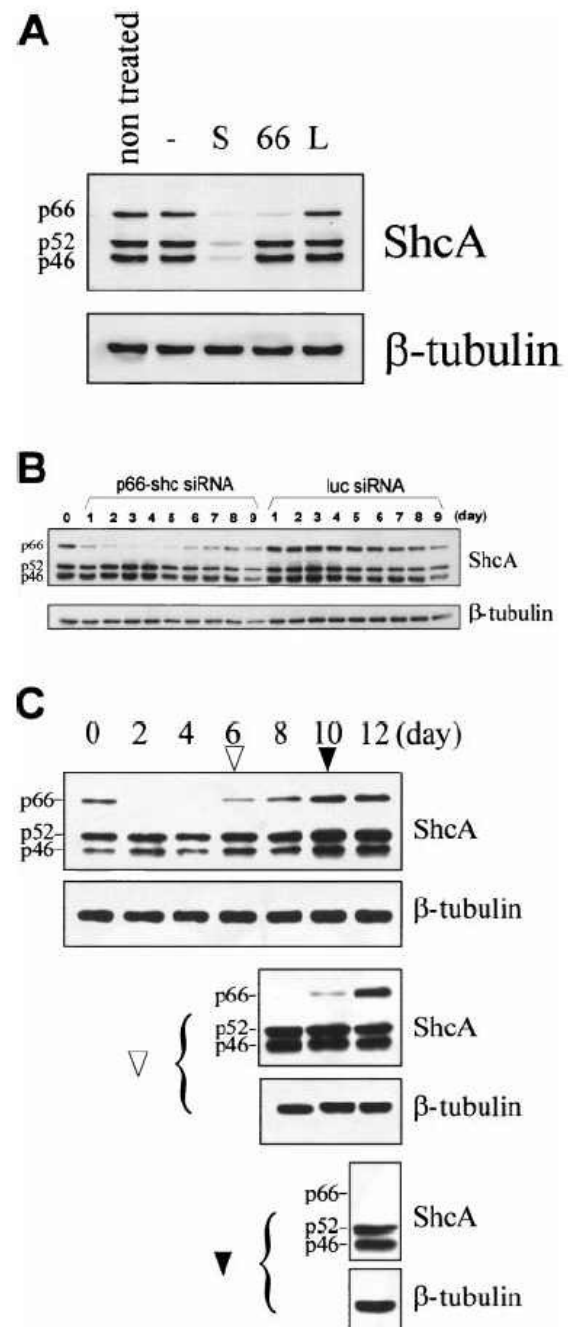
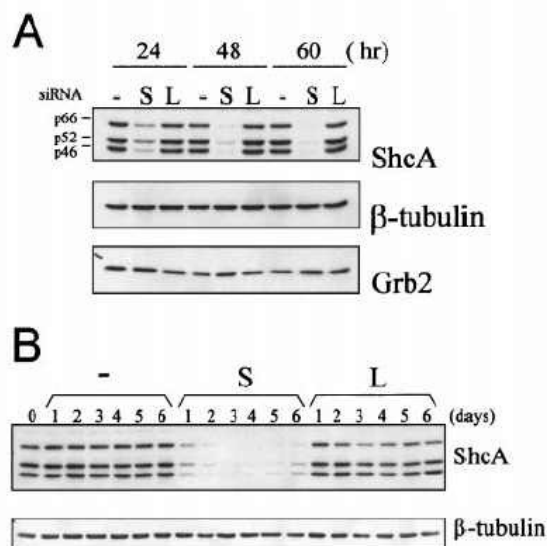
**Figure 1 Relationship between the ShcA gene, mRNA and protein.** The gene and mRNA are drawn to the same scale. Two transcription initiation sites are indicated by arrows. The boxes of the gene represent exons. Exon 0 that is under control of the p52/p46 promoter is ligated to exon 1 after splicing. Exons 1' and 1 are transcribed contiguously under the p66 promoter. Translation initiation sites are indicated both on the gene and mRNAs by triangles. The protein domains are demarcated and indicated: CH1 and 2, collagen homology 1 and 2; PTB, phosphotyrosine binding; and SH2, Src homology 2. The two siRNAs used in this work are indicated below p66 mRNA.

**Figure 2 Knock-down of ShcA in HeLa cells.**

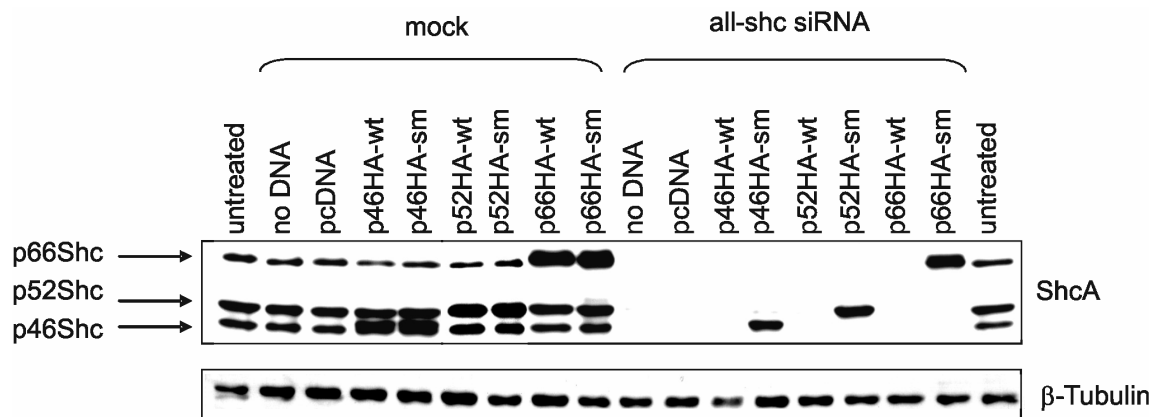
Cells were transfected using the oligofectAMINE™ reagent without siRNA (-) or with h/m-shc siRNA (S) and firefly luciferase siRNA (L). At different times after transfection, whole-cell extracts were prepared and analysed by Western blotting as described in the Experimental section. Membranes were probed for Shc,  $\beta$ -tubulin and Grb2. (A) Specificity; (B) time course.

**Figure 3 Isoform-specific knockdown**

(A) p66-selective knock-down. Cells were untreated or transfected without siRNA (-) or with h/m-shc siRNA (S), p66-shc siRNA (66) and control firefly luciferase siRNA (L). Whole-cell extracts were prepared 48h later and analysed for ShcA and  $\beta$ -tubulin levels as in



**Figure 2. (B) Time course.** Cells were transfected with p66-shc siRNA or luc siRNA and the levels of ShcA proteins were analysed at different times as above. (C) Repeated transfection. On days 6 (white triangle) or 10 (black triangle) after the first transfection, cells were transfected again with the same p66-shc siRNA. The levels of ShcA and control  $\beta$ -tubulin proteins were analysed at different times as above. Abbreviation: luc siRNA, firefly luciferase mRNA.



**Figure 4** Isoform-specific expression of ShcA

Cells were first transfected with no siRNA (mock) or with h/m-shc siRNA to down-regulate endogenous ShcA proteins. After 2 days, the cells were transfected with an empty expression vector, pCDNA3, or an expression vector for each isoform of wild-type (-wt) and silent mutant (-sm) ShcA. A day later, whole-cell extracts were prepared and analysed by Western blotting for the ShcA expression level. Membranes were blotted with polyclonal anti-ShcA and anti- $\beta$ -tubulin antibodies.

## DISCUSSION

In the present study we have shown that siRNA can efficiently, specifically and rapidly down-regulate the level of an endogenous protein in mammalian cells. The effect of siRNA was restricted to mRNAs containing a sequence identical with that of the siRNA used. That the primary action of siRNA on mRNA, which is most likely an endonucleolytic attack, does not propagate to other regions of the target mRNA was inferred from the following observations: (1) the effect of p66-shc siRNA was restricted to p66ShcA (Figure 3) and (2) h/m-ShcA siRNA targeted wild-type ShcA, but not mutant, mRNAs (Figure 4). In the first observation, expression of p52/p46 ShcA was not affected, although p66 and p52/p46 mRNAs shared sequence identity in most of the region 3' of the siRNA site (see Figure 1), indicating that the silencing signal does not propagate to regions of mRNA 3' to the siRNA. The second observation was with ectopically expressed ShcA mRNAs. In this experiment, sequences of wild-type ShcA mRNA and mutant ShcA mRNA for each isoform were identical, except for two nucleotides at the siRNA recognition site located in the middle of the mRNA. If the silencing signal did spread either 5' or 3' of the siRNA, ShcA expression from both wild-type and mutant mRNAs would have been suppressed. That expression from wild-type mRNAs but not from mutant mRNAs was suppressed strongly argues for stringent specificity of siRNA-mediated mRNA decay. This possibility was already suggested indirectly by Zamore et al. [18]. Using cell-free decay reactions containing insect cell lysates and dsRNA, they showed cleavage of target mRNA only within the region corresponding to the dsRNA. However, this analysis was only on the primary action of dsRNA-mediated mRNA cleavage and did not consider the possibility of signal amplification taking place *in vivo*. Indeed, results of experiments in an insect cell-free system [21] and *C. elegans* [22] have led to the suggestion

that long dsRNA molecules synthesized by RNA-dependent RNA polymerase are intermediates in RNAi that amplify and maintain the effect of siRNA. This implies 5' spreading of the silencing signal from siRNA. Our finding in HeLa cells that siRNA-mediated RNAi does not propagate to homologous regions of the target RNA suggests that this may not be the case in mammalian cells. Moreover, no endogenous RNA-dependent RNA polymerase has been reported in mammalian cells, except after RNA virus infection [23]. This may also explain why there is no systemic PTGS in mammals, which requires the amplification of siRNA, as has been often observed in other Kingdoms [16, 24]. Also, if amplification is involved in silencing in mammalian cells, antisense RNA oligonucleotides alone should serve as a primer for RNA-dependent RNA polymerase and thus be sufficient for gene down-regulation. We found that antisense RNA did not induce silencing (results not shown), in agreement with the previous report by Tuschl et al. [25]. Thus it may be a unique feature of mammalian cells that the silencing signal spreads neither 5' nor 3' of the siRNA, restricting the action of siRNA to mRNAs that carry a sequence identical with that of siRNA. An important implication of our results is that the combination of PTGS using siRNA and the isoform-specific expression of an homologous gene with silent mutations, which we call transient knockdown-in, causes the cell to express only one isoform, while keeping the levels of other isoforms very low. Using the transient knockdown-in method, it should be possible to examine the effect of various mutations of individual isoforms on cellular activity. The critical point in this method is that expression vectors should be designed so that they are not recognized by siRNA. Two mismatches in the mutated expression vectors were sufficient for them to be exempted from siRNA-mediated suppression (Figure 4). We used expression vectors for mouse ShcA in the present

study because there is a high degree of sequence similarity between mouse and human ShcA (90% mRNA sequences and 93% amino acid sequences for p66 isoform). Thus, instead of introducing mutations into expression vectors, it would have been possible to design a different siRNA with a sequence matching perfectly the endogenous human ShcA gene, but not the transfected mouse gene, and achieve similar results. The advantage of our approach, however, is that we can obtain isoform-specific ShcA expression in both human and mouse cells using the same set of probes.

The sequence of p66-shc siRNA is present in the primary transcripts of p52/p46 ShcA mRNA, but is spliced out of the mature mRNA (see Figure 1). The fact that only the p66 ShcA isoform was down-regulated by p66-shc siRNA strongly suggests that the site of action of siRNA is confined to the cytoplasm. If siRNA acted in the nucleus and triggered the decay of transcripts containing the corresponding sequence, the p52ShcA and p46ShcA proteins should have been equally down-regulated by the same p66-shc siRNA. Whether siRNA can enter the nucleus is not known. Even if it does so, it would be able to access to mRNA only after completion of its processing to mature mRNA. The response of cells to ShcA siRNA was rather fast and efficient; the target ShcA isoforms were already greatly decreased within 24h of transfection (>60%), and the protein levels remained low until 5 days after transfection. After 5 days, the reduced ShcA isoforms started to increase. There are two possibilities to account for this reappearance of the down-regulated isoforms: (1) adaptation of the cells to the siRNA by establishment of a resistant mechanism; or (2) dilution or degradation of siRNA with time. Because repeated transfection maintained or re-established the low levels of target ShcA isoform (Figure 3B), the latter simple dilution/degradation mechanism seems to apply. While a period of 5 days of down-regulation by a single transfection is long enough for some biochemical analysis, repeated transfection should allow long-term experiments. Repeated transfection will be necessary where the target protein has a slow turnover.

In summary, we have shown in mammalian cells that the site of action of siRNA-mediated mRNA degradation is confined to the cytoplasm and that the target mRNA is restricted to those mRNAs containing a sequence identical with that of the siRNA used. These specific features of siRNA-mediated gene knock-down can be employed over a short time period in conjunction with specific expression vectors to establish conditions for expression of the ShcA gene in an isoform-specific manner. This knockdown-in method should be applicable and useful for the study of genes expressed as multiple isoforms.

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## 2.2 Using siRNAs to study Shc function

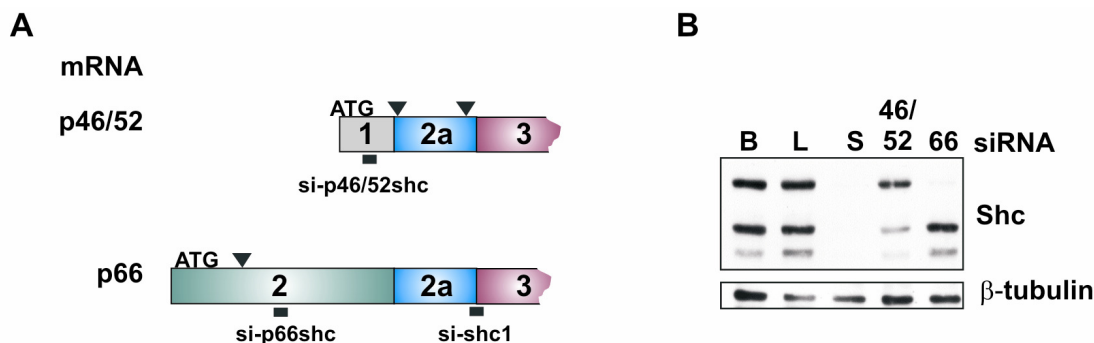
### 2.2.1 Isoform-specific knockdown of p46/52<sup>Shc</sup>

As described before, siRNA transfection allowed the knockdown of all Shc isoforms and, the specific knockdown of p66<sup>Shc</sup>. In the next step we generated siRNA to specifically knockdown p46/52<sup>Shc</sup>. The 3' end of the p46/52<sup>Shc</sup> mRNA contains exon 1 which is not translated and, more importantly, not present in the p66<sup>Shc</sup> mRNA (Fig 2.2.1A). Transfection of siRNA targeting this region (si-p46/52<sup>Shc</sup>) specifically reduced the protein levels of p46<sup>Shc</sup> and p52<sup>Shc</sup>, but did not change the amount of p66<sup>Shc</sup> (Fig 2.2.1B).

### 2.2.2 Growth inhibition upon Shc knockdown

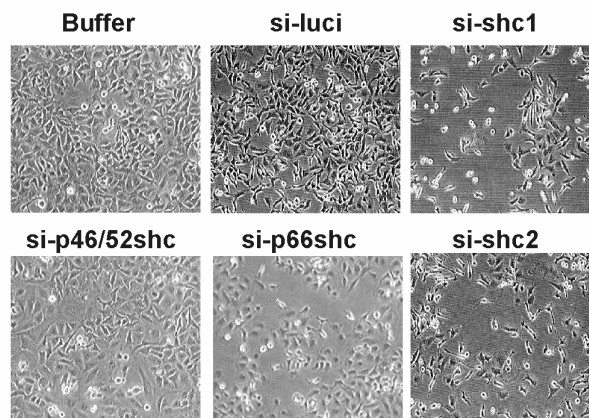
To develop the isoform-specific knockdown and the knockdown-in system, all experiments were done using 100 nM siRNA, based on an

earlier study involving siRNA-mediated knockdown of genes in human cells (Elbashir et al., 2001a). As a control for specificity, cells were transfected with siRNA targeting the luciferase gene (si-luci), which is not expressed in the cells, or with no siRNA (buffer) (Fig 2.2.1B). During the course of the experiments, we noticed that knockdown of Shc isoforms negatively influenced the growth of HeLa cells. While transfection without any siRNA (buffer) and with siRNA targeting the luciferase gene did not impair the proliferation of HeLa cells, transfection with Shc-targeting siRNAs reduced the growth of the cells (Fig 2.2.2-1A). This effect was strongest after knockdown of all Shc isoforms (si-shc1), and seemed to be milder after knockdown of only p66<sup>Shc</sup> or p46/p52<sup>Shc</sup>. To see whether the observed effect is due to Shc knockdown, a second siRNA (si-shc2) which targets a different region in the mRNA of all Shc isoforms was used. Upon transfection with si-shc2, HeLa cell proliferation was reduced to a similar extent to si-shc1, when measured using an MTT proliferation assay (Fig 2.2.2-1B).

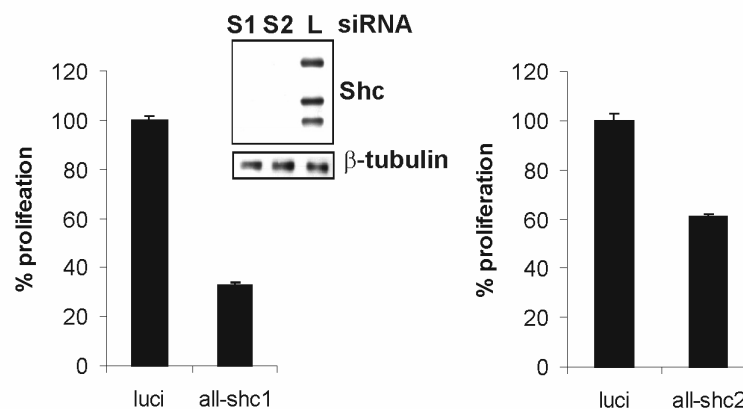


**Figure 2.2.1: Isoform-specific knockdown of Shc** **A.** Schematic representation of p46/52<sup>Shc</sup> and p66<sup>Shc</sup> mRNA. The triangles point to the ATG start codons, and the black boxes indicate the site targeted by the siRNA. **B.** HeLa cells were transfected with different siRNAs and collected three days later. Total cell lysate was used for Western blot analysis to probe for Shc and  $\beta$ -tubulin levels. **B:** buffer; L: si-luci; S: si-shc1; 46/52: si-p46/52shc; 66: si-p66shc.

A



B



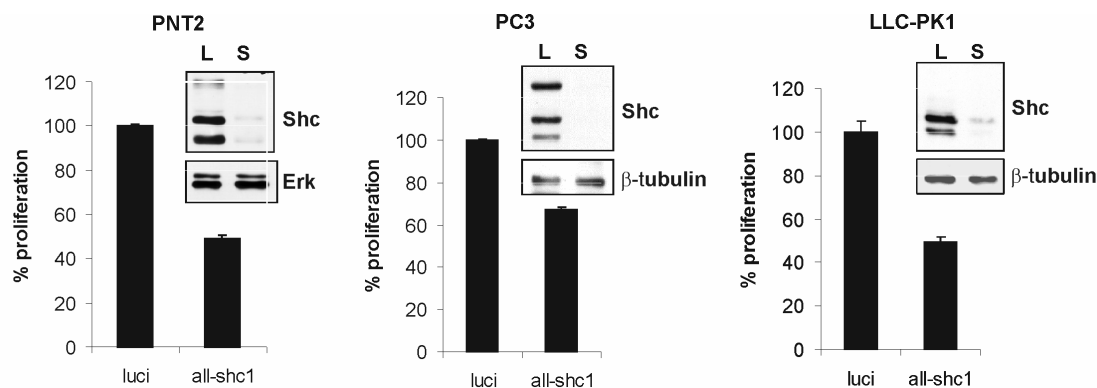
**Figure 2.2.2-1: Transfection of *Shc* siRNA inhibits growth in HeLa cells.** **A.** HeLa cells were transfected with different siRNAs as indicated. Three days later pictures were made using light microscopy. **B.** HeLa cells were transfected with si-shc1 (S1), si-shc2 (S2), or si-luci (L) and proliferation was measured after three days using an MTT proliferation assay: additionally, total cell lysate was used for Western blot analysis.

We next investigated whether this growth inhibition is a general effect which can also be observed in other cell types. As depicted in Fig. 2.2.2.-2, knockdown of all *Shc* isoforms inhibited proliferation in PNT2 cells, PC3 cells, and LLC-PK1 cells that do not express p66<sup>Shc</sup>.

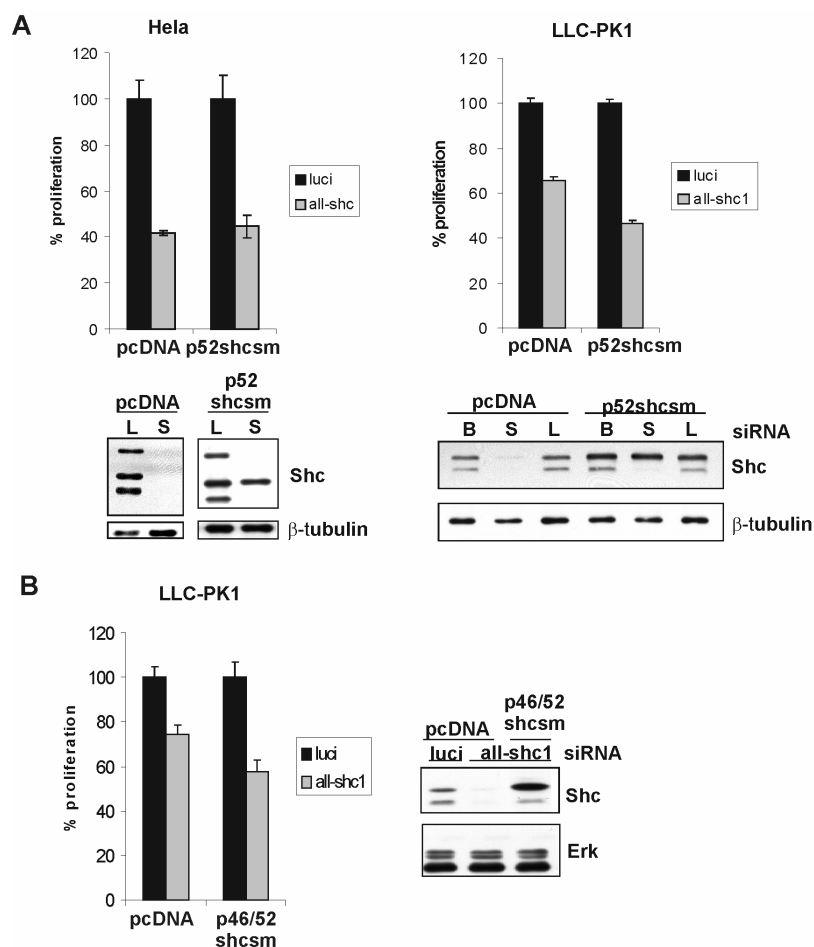
To investigate which *Shc* isoform was responsible for the growth inhibitory effect and would be able to rescue it, the knockdown-in system was used. LLC-PK1 and HeLa cells were generated to stably express single *Shc* isoforms which carry a silent mutation in the region targeted by the siRNA. These cells were subsequently used to knockdown the

endogenous *Shc* proteins, and proliferation was measured by MTT assay. Expression of p52<sup>Shc</sup> did not rescue proliferation in HeLa or LLC-PK1 cells (Fig 2.2.2-3A). Moreover, expression of siRNA-resistant p46<sup>Shc</sup> in addition to p52<sup>Shc</sup> also did not prevent the growth-inhibitory effect caused by the siRNA in LLC-PK1 cells, although the *Shc* expression level was completely rescued (Fig 2.2.2-3B). These experiments demonstrated that the effect of siRNA transfection on proliferation was not dependent on *Shc* protein levels, but presented rather an unspecific response.





**Figure 2.2.2-2: Transfection of *Shc* siRNA inhibits growth in PNT2, PC3, and LLC-PK1 cells.** Cells were transfected with *si-shc1* (S) or *si-luci* (L), and three days later proliferation was measured using an MTT assay and total cell lysate was analyzed by Western blotting.

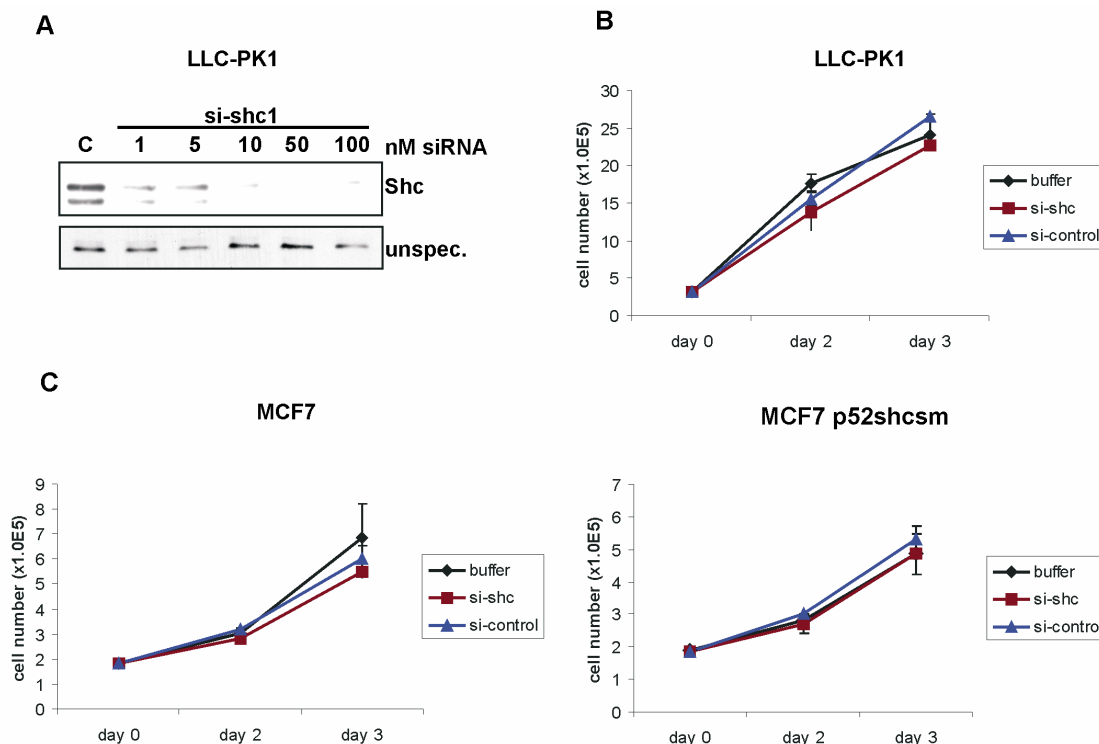


**Figure 2.2.2-3: Growth inhibition caused by *si-shc* is an unspecific effect.** Stable HeLa or LLC-PK1 cell lines expressing an empty vector or silent mutants of HA-p52<sup>shc</sup> (p52shcsm) (**A**) and HA-p46/52<sup>Shc</sup> (only LLC-PK1) (**B**) were prepared and transfected with siRNA targeting all *Shc* isoforms (S) or *luci* siRNA (L). After three days of transfection, proliferation was measured using an MTT assay and total cell lysate was analyzed by Western blotting.

To avoid unspecific events for future experiments, the minimum amount of siRNA sufficient for efficient knockdown was determined. Fig. 2.2.2-4A shows that knockdown using only 10 nM siRNA reduced Shc protein levels as effectively as knockdown using 100 nM siRNA. Moreover, almost no effect on proliferation was observed after transfection with 10 nM si-shc when compared

to buffer transfection or transfection with a scrambled control si-RNA in LLC-PK1 and MCF7 cells (Fig. 2.2.2-4B, C).

In conclusion, to study the function of proteins using siRNA only low concentrations of siRNA should be used to avoid unspecific effects, and rescue experiments should be performed to ensure specificity.



**Figure 2.2.2-4 Knockdown of Shc using 10 nM siRNA.** **A.** Indicated amounts of si-shc1 were transfected into LLC-PK1 cells and, three days after transfection, total cell lysate was analyzed by Western blotting. **B, C.** LLC-PK1, MCF7, and a stable MCF7 cell line expressing HA-p52<sup>Shc</sup> were transfected with buffer, si-shc1, or si-control. Proliferation was measured by counting cells with the Vi-CELL analyzer.

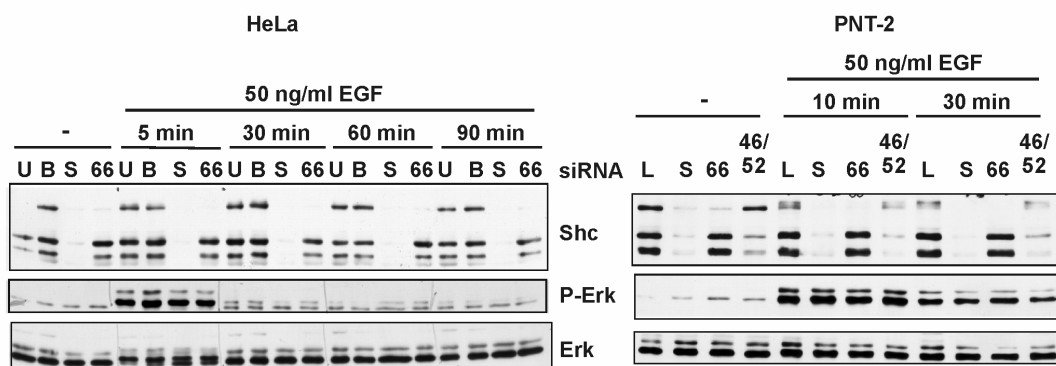
## 2.3 Role of Shc in EGF-induced signaling in epithelial cells

### 2.3.1 Role of Shc in EGF-induced Erk activation

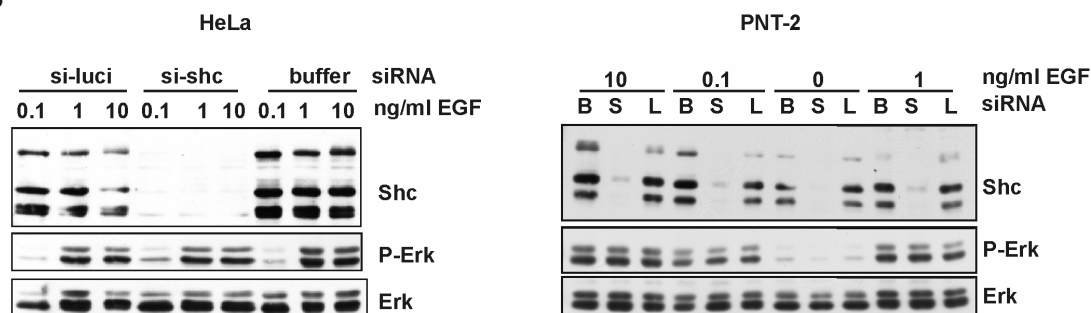
Shc has been shown to be involved in EGF-induced Erk activation (1.1.2). However, the contribution of each Shc isoform to this event has not been investigated. In addition, it is still unclear whether p66<sup>Shc</sup> influences Erk activation in a dominant negative manner. The isoform-specific knockdown and the knockdown-in system provide perfect tools for investigating this question. Surprisingly,

knockdown of either all Shc isoforms or only p66<sup>Shc</sup> did not affect EGF-induced Erk activation at any timepoint in HeLa cells (Fig 2.3.1A right panel). In addition, knockdown of any Shc isoform did not influence EGF-induced Erk activation in the epithelial cell lines PNT2 (Fig 2.3.1B left panel), PC3, and LLC-PK1 (data not shown). Results with Shc-deficient MEFs suggest that Shc sensitizes cells to low amounts of growth factors. Therefore, siRNA-transfected HeLa and PNT2 cells were treated with low concentrations of EGF in the next experiment. EGF-induced Erk activation was reduced by low amounts of EGF; however, Shc knockdown had no influence in both HeLa and PNT2 cells (Fig 2.3.1B).

**A**



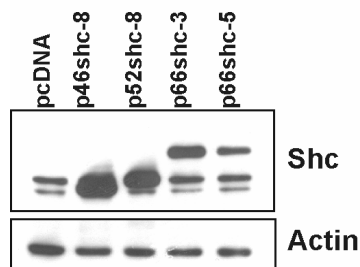
**B**



**Figure 2.3.1: Effect of Shc knockdown on EGF-induced Erk activation.** Cells were transfected with siRNA and treated three days later with 50 ng/ml EGF for the indicated time (**A**) or for 10 min with the indicated EGF concentration (**B**). Total cell lysate was subjected to Western blot analysis. U: untreated; B: buffer; L: si-luciferase; S: si-shc1; 66: si-p66<sup>Shc</sup>; 46/52: si-p46/52<sup>Shc</sup>.

### 2.3.2 Effect of p66<sup>Shc</sup> on EGF-driven proliferation and cell survival

MCF7 epithelial breast cancer cells express p66<sup>Shc</sup> at a very low level. Some reports suggest that reduced p66<sup>Shc</sup> expression may play a role in breast cancer, which is often driven by signaling from the EGFR family (Stevenson and Frackelton, 1998; Xie and Hung, 1996). Since p66<sup>Shc</sup> might negatively influence Erk signaling downstream of EGFR family members, it is intriguing to speculate that p66<sup>Shc</sup> reduces EGF-driven proliferation or survival. To investigate p66<sup>Shc</sup> function in MCF7 cells, stable cell lines overexpressing HA-p66<sup>Shc</sup> were generated. In addition, empty vector-expressing cells or cell clones overexpressing HA-p46<sup>Shc</sup> or HA-p52<sup>Shc</sup> were made as a control (Fig 2.3.2-1).

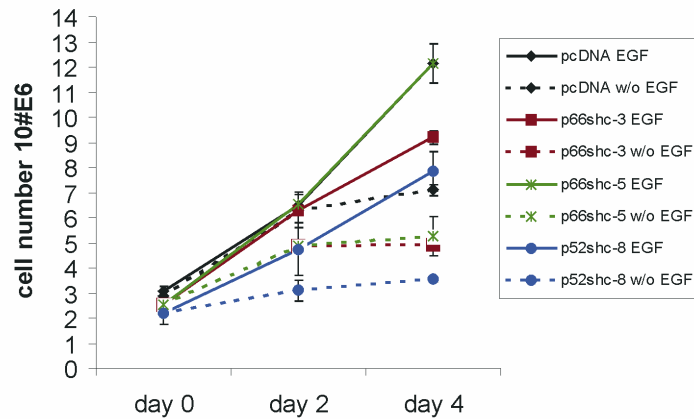


**Figure 2.3.2-1: MCF7 cells stably overexpressing Shc isoforms.** MCF7 cells were stably transfected to overexpress HA-p46<sup>Shc</sup>, HA-p52<sup>Shc</sup>, and HA-p66<sup>Shc</sup>. The clones depicted here were used for further experiments.

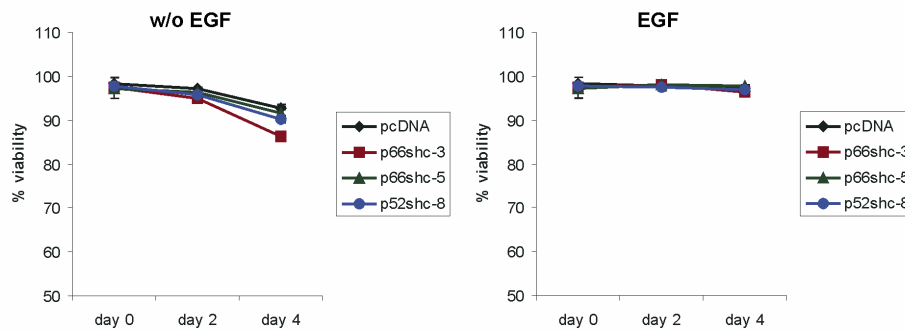
The responsiveness to EGF-driven proliferation or viability of these cell clones was then investigated. Proliferation under starvation conditions without EGF was slowed down in all cell clones (Fig. 2.3.2-2A). The addition of 50 ng/ml EGF to the starvation medium enhanced proliferation in all cell clones. Although these cell clones exhibited different proliferation rates, there was no consistency with respect to

p66<sup>Shc</sup> expression. We also tested other clones (data not shown), but the proliferation rate seemed to be clone-dependent, rather than dependent on the expression of Shc isoforms. The same result was observed for cell viability, which started to decline 4 days after starvation in the absence of EGF. However, EGF treatment restored cell viability in all cell clones, independent of their p66<sup>Shc</sup> expression level (Fig. 2.3.2-2B), suggesting that neither EGF-induced proliferation nor cell survival was influenced by the expression of p66<sup>Shc</sup> in MCF7 cells.

A



B



**Figure 2.3.2-2: Effect of p66<sup>Shc</sup> overexpression on EGF-mediated proliferation and survival.** Stably transfected MCF7 cells expressing empty vector, HA-p66<sup>Shc</sup>, or HA-p52<sup>Shc</sup> were seeded, and from the next day (day 0) starved in medium containing 0.1 % FCS. Alternatively, 50 ng/ml EGF was added to the starvation medium. At days 0, 2, and 4 after starvation, cells were counted and viability was measured using trypan blue exclusion with VI-CELL analyzer.

## 2.4 Research Publication (under review)

### INDUCTION OF UPA GENE EXPRESSION BY THE BLOCKAGE OF E-CADHERIN VIA SRC- AND SHC-DEPENDENT ERK SIGNALING

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Running title: Loss of E-cadherin function induces uPA

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Loss of E-cadherin-mediated cell-cell adhesion and expression of proteolytic enzymes characterize the transition from benign lesions to invasive, metastatic tumor, a rate-limiting step in the progression from adenoma to carcinoma *in vivo*. A soluble E-cadherin fragment recently found in the serum and urine of cancer patients has been shown to disrupt cell-cell adhesion and to drive cell invasion in a dominant-interfering manner. Physical disruption of cell-cell adhesion can be mimicked by the function-blocking antibody Decma. We have shown previously in MCF7 and T47D cells that urokinase-type plasminogen activator (uPA) activity is upregulated upon disruption of E-cadherin-dependent cell-cell adhesion. We explored the underlying molecular mechanisms and found that blockage of E-cadherin by Decma elicits a previously undescribed signaling pathway downstream of E-cadherin that leads to Src-dependent Shc and Erk activation and results in uPA gene activation. siRNA-mediated knockdown of endogenous Shc and subsequent expression of single Shc isoforms revealed that p46<sup>Shc</sup> and p52<sup>Shc</sup> but not p66<sup>Shc</sup> were able to mediate Erk activation. A parallel pathway involving PI3K contributed partially to Decma-induced Erk activation. This report describes that disruption of E-cadherin-dependent cell-cell adhesion induces intracellular signaling with the potential to enhance tumorigenesis and, thus, offers new insights into the pathophysiological mechanisms of tumor development.

Tumor cell invasion, a key event of metastatic progression, requires spreading of tumor cells from the primary tumor. This is strongly dependent on the loss of homotypic cell-cell adhesion. E-cadherin is an important component of the cell-cell adhesion complex and required for the formation of epithelia in the embryo and the maintenance of the polarized epithelial structure in the adult (2). As a single-span transmembrane-domain glycoprotein, E-cadherin mediates cell-cell adhesion via calcium-dependent homophilic interaction of its extracellular domain (3). Proteins such as p120-catenin,  $\alpha$ -catenin and  $\beta$ -catenin assemble the cytoplasmic cell adhesion complex on its intracellular domain and link E-cadherin indirectly to the actin cytoskeleton (3).

E-cadherin is considered to be an important tumor suppressor (2,4). *In vitro* studies have clearly established a direct correlation between a defect in functional E-cadherin expression at the cell surface and the acquisition of an invasive phenotype (3). Moreover, a partial if not complete reversal of the invasive phenotype could be achieved by ectopic expression of E-cadherin (3,5). While E-cadherin expression is maintained in most differentiated carcinomas, there is a strong correlation in several types of cancer, including breast, gastric, prostate, and colon carcinoma, between loss of E-cadherin expression and aggravated phenotypes, e.g. metastasis and malignancy leading to a poor survival rate (4). Loss of E-cadherin-mediated cell-cell adhesion occurs through various mechanisms, such as downregulation of E-cadherin expression via promoter hypermethylation (6), transcriptional repression (7), E-cadherin gene mutation (7), modification of  $\beta$ -catenin (8), or the cleavage of E-cadherin by

matrix metalloproteases (MMPs) (9). Cleavage of E-cadherin results not only in the disruption of cell-cell adhesion but also in a soluble 80-kDa E-cadherin fragment that itself disrupts cell-cell adhesion in a dominant-interfering manner, thereby promoting tumor progression (10). However, the intracellular processes subsequent to disruption of cell-cell adhesion remain elusive. Tumor metastasis involves, in addition to the loss of cell-cell adhesion, degradation of the extracellular matrix. MMPs and urokinase-type plasminogen activator (uPA) are known to be involved in extracellular matrix degradation. Moreover, increased expression of uPA is directly related to higher tumor growth and metastasis (1). Several analyses have already made it clear that the expression of E-cadherin and the expression of MMPs are inversely correlated (11,12) and that E-cadherin-dependent cell-cell contact regulates the expression of MMPs and uPA *in vitro* (13-15). However, the underlying molecular mechanisms are not yet fully understood. Expression of genes for these proteolytic enzymes can be induced by various stimuli, including growth factors and integrin ligation, and has often been shown to be Erk-dependent (16-18). The adaptor protein ShcA, which is referred to here as Shc, is involved in coupling receptor and non-receptor tyrosine kinases to the Ras/extracellular regulated kinase (Erk) pathway (19). Shc is expressed in three different isoforms, p46<sup>Shc</sup>, p52<sup>Shc</sup> and p66<sup>Shc</sup>, but only the two smaller isoforms seem to be involved in Erk activation (20). Receptor tyrosine kinases activated by tyrosine phosphorylation recruit and phosphorylate these Shc isoforms. This creates a binding site for Grb2 and results in the recruitment of the Grb2/Sos complex to the vicinity of Ras, where Sos acts as a GTP exchange factor for Ras.

An increasing body of evidence suggests that cadherins act at the cellular level as adhesion-activated cell signaling receptors (3). Indeed, homophilic ligation of the E-cadherin ectodomain induces activation of several signaling molecules, such as Rho-family GTPases (3), MAPKs (22) and PI3K (3). These signals are believed to regulate dynamic organization of the actin cytoskeleton and the activity of the cadherin/catenin apparatus to support stabilization of the adhesive contact (23). Several studies have suggested functional interdependence of cadherins and receptor tyrosine kinases with respect to their signaling capacities. It has been shown that initiation of *de novo* E-cadherin-mediated adhesive contacts can

induce ligand-independent activation of the EGF receptor (EGFR) and subsequent activation of (Erk) (22) (14). Moreover, it was shown that the E-cadherin adhesive complex can be linked directly to EGFR via the extracellular domain of E-cadherin and negatively regulate receptor tyrosine kinase signaling in an adhesion-dependent manner (24).

We have shown previously that disruption of E-cadherin-dependent cell-cell adhesion with the function-blocking antibody Decma (also termed Uvomorulin antibody or anti- Arc-1) results in disruption of cell-cell adhesion of T47D and MCF7 breast cancer cells (25). The loss of the epithelial morphology was associated with an increased secretion of uPA into the extra cellular milieu. Furthermore, Decma treatment induced invasiveness into collagen which was inhibited by the addition of uPA antibodies. The enhanced uPA secretion was dependent on transcription (25). It appears therefore that disruption E-cadherin-dependent cell-cell adhesion initiates signaling events leading to the uPA gene. However, the nature of these signaling events has remained largely unknown. Since both disruption of E-cadherin-dependent cell cell-adhesion and the expression of uPA are causally involved in tumor progression, the understanding of these underlying intracellular events is of paramount importance. In the present study, we explored the signaling pathway linking perturbation of E-cadherin-dependent cell-cell adhesion to the activation of Erk and the uPA gene expression.

## MATERIALS AND METHODS

**Reagents** - Decma and HA antibodies were used as a hybridoma supernatant (containing approximately 40-50 µg/ml antibody) dialyzed against DMEM ( $M_r$  cutoff  $1.2 \times 10^4$ ). The Decma hybridoma cell line was kindly provided by D. Vestweber and R. Kemler. The dialyzed counterpart was used for control treatments. Unless indicated otherwise, cells were treated for 30 min with Decma supernatant (40-50 µg/ml antibody) or control supernatant. Anti-Shc polyclonal, anti-Grb2, anti-E-cadherin monoclonal (used for Western analysis and immunostaining), and antiphospho-Src (Tyr-416) polyclonal antibodies were obtained from Transduction Laboratories. Antiphospho-PKB (Ser 473) and antiphospho-Erk polyclonal antibodies were from Cell Signaling, and anti-Erk polyclonal, anti E-cadherin (sc-8426) (E-

cad2) and anti-EGFR (sc-101) monoclonal antibody were obtained from Santa Cruz. Mouse monoclonal HA antibodies (12CA5) used for Western blotting or immunoprecipitation were purified on a protein A-Sepharose column and monoclonal antibodies against phosphotyrosine (4G10) were used as hybridoma supernatant. Anti-Src mouse monoclonal antibody (clone 327) was a gift from K. Ballmer-Hofer (Paul Scharrer Inst.). SB203580 and CGP77675 were kindly provided by E. Blum (Novartis AG), Wortmannin, UO126 and Y27634 were obtained from Calbiochem, LY294002 and Cytochalasin D (CytD) was from Sigma, SP600125 was obtained from Biomol, and TPA, horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies, ECL reagent, protein A and G-Sepharose were from Amersham Bioscience.

**Cells and transfection** - MCF7 and T47D cells were cultured in DMEM/HAMs F12 (Invitrogen) supplemented with 10% FCS, 0.2 mg/ml streptomycin, and 50 units/ml penicillin at 37°C in a humidified incubator with 5% CO<sub>2</sub>. For plasmid transfection, T47D cells (0.7×10<sup>6</sup>/well) were seeded in 6-well tissue culture plates and incubated overnight. Plasmid DNA (1 µg) was transfected using 5 µl Lipofectamine 2000 according to the manufacturer's instructions. Fugene 6 was used to transfect MCF7 cells (0.5×10<sup>6</sup>/well). Plasmid DNA (1 µg) was incubated with Fugene 6 (ratio 2:3) for 20 min and the whole mixture was added to the cells, which were then incubated overnight at 37°C. For siRNA transfection, T47D cells (0.18×10<sup>6</sup>/well) and MCF7 cells (0.12×10<sup>6</sup>/well) were seeded and transfected the next day with 10 nM siRNA as described (26) using 5 µl and 3 µl Oligofectamine, respectively. MCF7 cells expressing siRNA against E-cadherin or mouse NCAM were generated by the stable transfection of the pSuper retro vector containing the respective sequences. These cell lines were kindly provided by F. Lehembre and G. Christofori and details will be published elsewhere.

**Plasmids and siRNAs** - Construction of expression vectors for HA-tagged full-length mouse p46<sup>shc</sup>, p52<sup>shc</sup>, and p66<sup>shc</sup> and the introduction of silent mutations by site-directed mutagenesis were described previously (26). The uPA-reporter plasmid pGL-2-puPA-4.6 was described previously (27). The following 21-mer oligoribonucleotide pairs (siRNAs) were used: shc siRNA nt 677–697 (in the protein tyrosine binding domain), 5'-CUA CUU GGU UCG GUA CAU GGG-3' and 5'-CAU GUA CCG

AAC CAA GUA GGA-3'; control siRNA 5'-GUA CCU GAC UAG UCG CAG AAG-3' and 5'-UCU GCG ACU AGU CAG GUA CGG-3'. The specificities of these sequences were confirmed by blasting against the GenBank/EMBL database.

**Immunoprecipitation and Western blot analysis** - Immunoprecipitation and Western blotting were performed as described (28).

**RNA isolation and Northern blot analysis** - MCF7 cells (0.5×10<sup>6</sup>/well) were seeded in 6-well tissue culture plates. After 2 days, cells were treated as indicated, total RNA was isolated and 10 µg aliquots subjected to Northern blot analysis as described (27).

**Immunofluorescence** - Immunostaining and microscopy were carried out as described previously (28). Briefly, cells were cultured on a cover slip, fixed in 1 ml of pre-warmed 3% paraformaldehyde in PBS for 20 min at room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, blocked with 5% normal goat serum for 20 min, incubated with anti-E-cadherin antibody (1:200) for 2 h, washed 3 times with PBS, incubated 45 min with Alexa 488 (1:500) and washed again 3 times. Finally, the cover slips were mounted on glass slides with Fluoromount-G (Southern Biotechnology Associates Inc). Fluorescence was visualized with a Zeiss Axioplan fluorescence microscope (63X oil objective with, numerical aperture of 1.4) and all images were captured using Axiovision 3.0 software.

**Reporter gene assay (dual-luciferase-assay)** - Cells (5×10<sup>5</sup>) were plated in a 6-well dish to be co-transfected the next day with the reporter plasmid and the Renilla control plasmid using Fugene 6. One day after transfection, cells were pretreated for 45 min with the indicated inhibitors and afterwards with Decma for 5 h before harvesting. Luciferase expression was measured according to the given protocol (Dual-Luciferase Reporter Assay System, Promega) and normalized against Renilla expression.

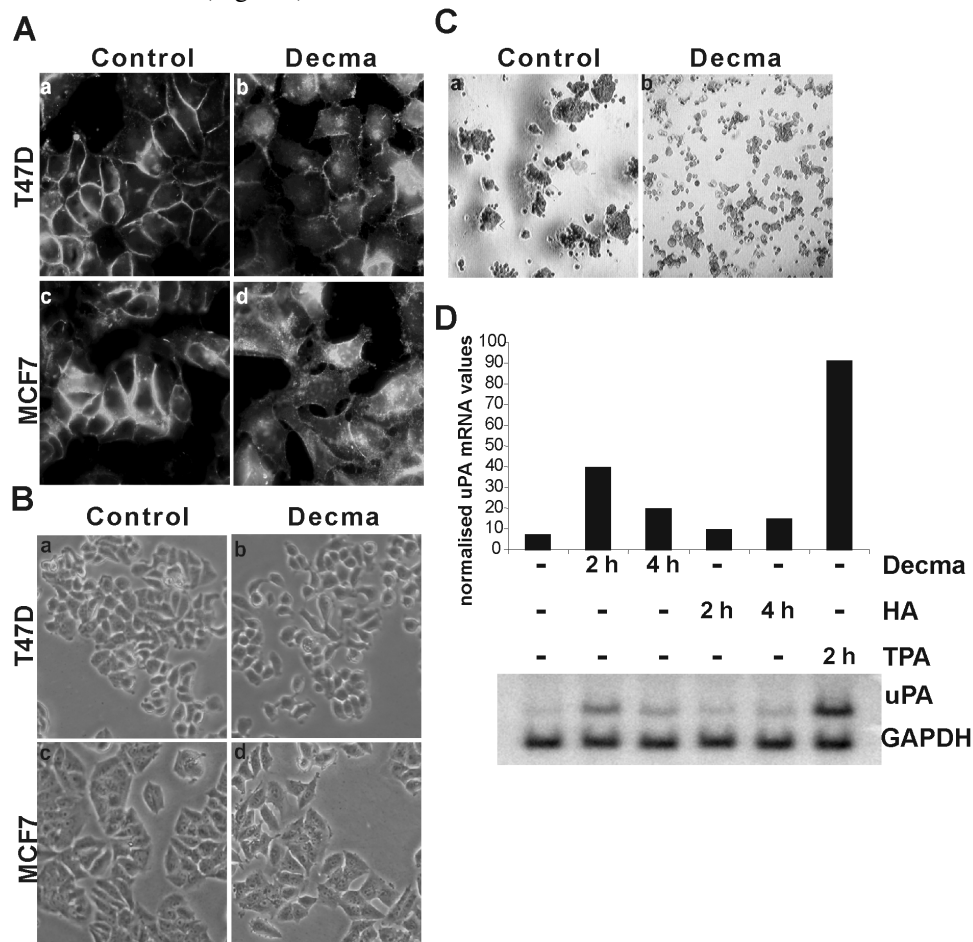
## RESULTS

**Decma treatment disrupts E-cadherin-dependent cell-cell adhesion and induces uPA gene expression** - Under normal growth conditions, T47D and MCF7 breast cancer epithelial cell lines grow very compact and E-cadherin was concentrated at the border of the cell-cell interaction, corresponding to typical adhesive junction localization (Fig. 1A, a and c).

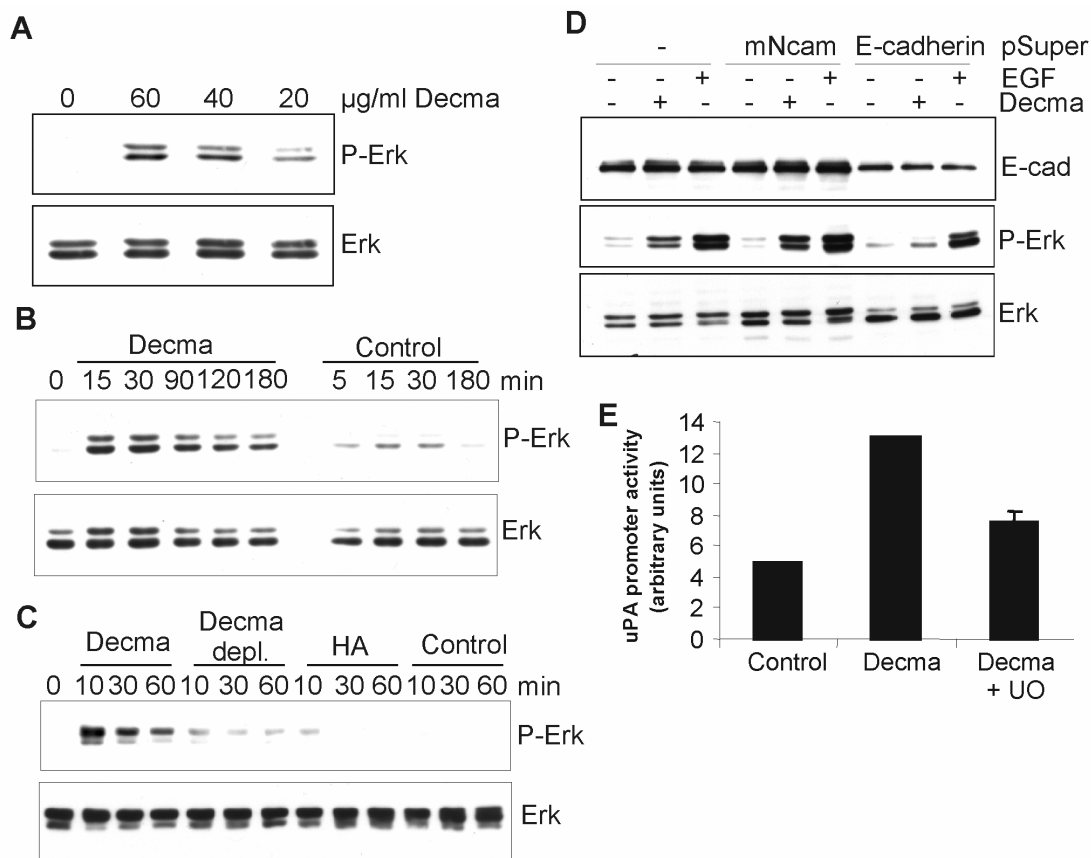


As described previously (25), Decma treatment destroyed tight cell-cell interaction, resulting in disruption of the epithelial layer (Fig 1A, b and d) and acquisition of a scattered phenotype (Fig. 1B). In addition, E-cadherin disappeared from the plasma membrane and was redistributed into the cytoplasm, suggesting its internalization (Fig. 1A, b and d). The loss of functional cell-cell adhesion became more apparent when MCF7 cells were grown in Matrigel. Decma treatment led to the resolution of compact cell clusters and dissociation of the cells (Fig. 1C). To determine

whether the disruption of cell-cell adhesion by Decma influenced expression of the uPA gene, we examined change in uPA mRNA levels. Northern blot analysis showed only barely detectable levels of uPA mRNA under normal growth conditions. However, an increase in uPA mRNA levels was observed already at 2 h after Decma treatment (Fig. 1D), whereas the control treatment (HA supernatant) had no effect. As a positive control, cells were treated with TPA, a potent inducer of uPA gene expression (16).



**Fig 1. Effects of Decma treatment on E-cadherin distribution, cell scattering and uPA expression.** A, T47D cells (a and b) and MCF7 cells (c and d) were treated for 4 h with control or Decma supernatant and immunostained with anti-E-cadherin antibody recognizing the cytoplasmic part of E-cadherin. B, T47D cells (a and b) and MCF7 cells (c and d) were grown for 2 days to 60-70% confluence and then treated with control or Decma supernatant for 6 h before recording. C, MCF7 cells were grown overnight in Matrigel in the presence of control or Decma supernatant and stained with crystal violet to visualize cells before recording. D, MCF7 cells were treated with Decma and HA supernatant or 100 ng/ml TPA as indicated and subjected to Northern blot hybridization analysis for uPA and GAPDH mRNA levels. The uPA mRNA levels were normalized against GAPDH mRNA.



**Fig 2. Role of Erk in Decma-induced uPA upregulation.** *A, B*, T47D cells were treated for 30 min with different amounts of supernatant (*A*) or for different time periods (*B*) and total cell lysate was subjected to Western blot analysis for phospho-Erk levels. *C*, Comparison of phospho-Erk levels induced by different supernatants. MCF7 cells were treated for 30 min with Decma supernatant (Decma), Decma supernatant after Decma-depletion (Decma-depl.), anti-HA antibody-containing supernatant (HA) or control supernatant before analyzing the total cell lysates by Western blotting. For Decma-depleted supernatant, Decma supernatant was incubated with Protein A beads rotating overnight to pull down the antibody. *D*, MCF7 cells stably transfected with a pSuper retro-vector to express siRNA targeting E-cadherin or mouse-specific NCAM were treated for 30 min with Decma, or 10 min with 50 ng/ml EGF, and total cell lysates were subjected to Western blot analysis for phospho-Erk status. *E*, MCF cells were co-transfected with a luciferase construct under the control of the uPA promoter and the *Renilla* plasmid overnight. Cells were then pretreated for 45 min with 10 µM UO126 (UO) as indicated and subsequently for 5 h with Decma or control supernatant before harvesting. Luciferase activity was measured and normalized against *Renilla*.

**Decma-induced uPA gene expression is dependent on Erk activation** - We and others have shown that activation of Erk plays an important role in uPA gene expression (16,29). To determine whether Decma treatment caused activation of Erk, we investigated the phosphorylation status of Erk. Western blot analysis revealed a dose-dependent increase in Erk phosphorylation upon Decma treatment (Fig. 2A). This phosphorylation peaked 10-15 min after Decma treatment and declined slowly, but remained at substantial levels for more than 3 h (Fig. 2B). Low and transient increase in Erk phosphorylation observed in control and HA-treated cells (Fig. 2C) may be a response to

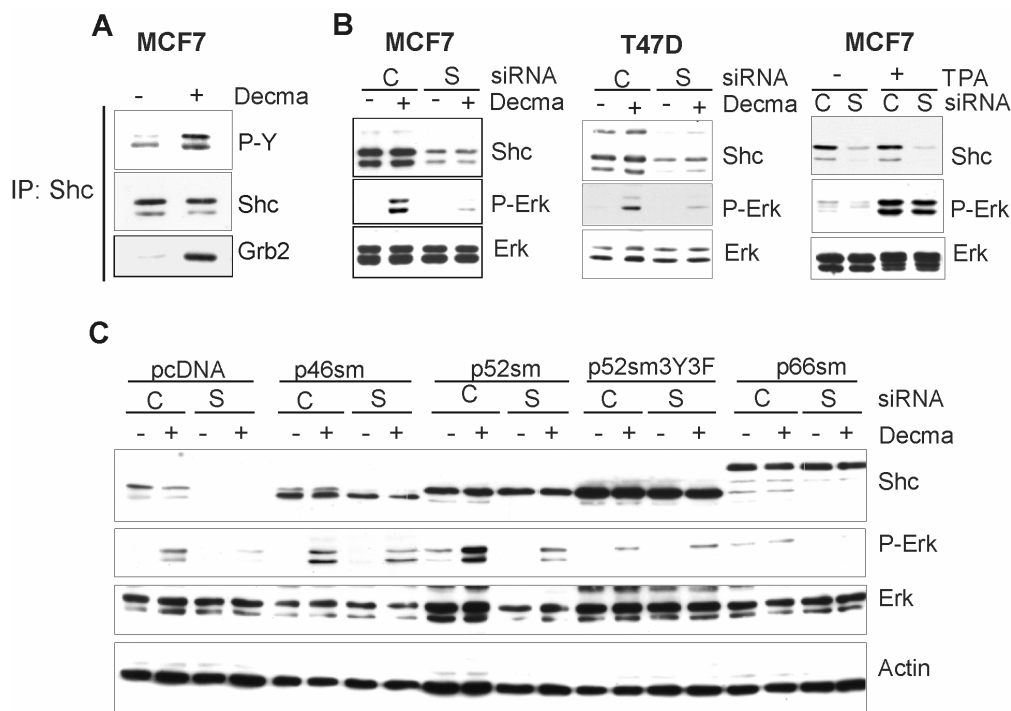
medium change, which is known to activate Erk. To test whether the observed Erk phosphorylation was due to the blocking activity of Decma, we depleted Decma antibody molecules with Protein A-Sepharose. Treatment of MCF7 cells with this Decma-depleted conditioned medium had no pronounced effect on scattering (data not shown) or marked Erk phosphorylation (Fig. 2C). To test whether the observed Erk activation is a result of an interaction between Decma and E-cadherin, we examined the effect of the Decma-conditioned medium on cells expressing low amounts of E-cadherin using an MCF7 cell line stably transfected with a pSuper retro vector expressing

an E-cadherin-specific siRNA. As a control, cells were stably transfected with a pSuper vector expressing siRNA to target mouse-specific NCAM, an mRNA that is not expressed in this cell line. Although, the knockdown of E-cadherin was not complete, Decma-induced Erk activation was markedly lower under these conditions than in non-transfected or control cells (Fig. 2D). Thus the effect of Decma on Erk activation depends on the presence of the E-cadherin protein. EGF-induced Erk activation was not affected in any of these cell lines. To ascertain that Erk activation was a result of disruption of cell-cell adhesion and not merely of binding of an antibody to E-cadherin or to any given surface molecule, we treated MCF7 cells with a second anti-E-cadherin (E-cad2) and an anti-EGFR antibody. Both antibodies recognize the extracellular part of their respective proteins. In contrast to Decma, however, none of them induced disruption of cell-cell adhesion and scattering (data not shown). Western blot analysis revealed that in contrast to Decma treatment, neither treatment with the E-cad2 antibody nor the EGFR antibody induced Erk activation (SupFig). Taken together, these results suggest that the observed Erk activation was specific for the disruption of cell-cell adhesion induced by blocking of E-cadherin via Decma. To find out whether Decma-induced Erk activation is necessary for enhanced uPA gene expression, we examined the effect on uPA promoter activity of the inhibitor UO126, which blocks MEK1, the upstream kinase of Erk. Transient transfection assays showed that Decma treatment strongly enhances uPA promoter activity, which was efficiently suppressed by pretreatment of the cells with UO126 (Fig. 2E). These results indicate that Decma treatment activates the uPA promoter through a signaling pathway involving Erk.

*Shc is necessary for Decma-induced Erk activation* - Activation of Erk by various extracellular signals is often preceded by Shc phosphorylation. Accordingly, we examined Shc activation, as indicated by its tyrosine phosphorylation, and its association with Grb2. Both Shc activation and its Grb2 association increased after Decma treatment in MCF7 cells (Fig. 3A) and T47D cells (data not shown). RNAi experiments were performed to examine whether Shc activation is causally linked to Erk activation. Knockdown of all Shc isoforms by siRNA strongly decreased Erk phosphorylation in both MCF7 and T47D cell lines, while control siRNA had no effect (Fig. 3B). The observed

impact on Erk activity was not a general effect of siRNA on Erk signaling since TPA-induced Erk activation was not affected by the same siRNA (Fig. 3B, right panel). To further test whether the inhibition of Erk activation was caused by the reduction in Shc protein, rescue experiments were performed using the siRNA-mediated knockdown-in approach (26). MCF7 cells were stably transfected with plasmids encoding single Shc isoforms, which carry silent mutations at the targeting site of the siRNA. These cell lines were further used for siRNA transfection to knockdown the endogenous proteins without affecting the silent mutant isoform. Fig. 3C shows that knockdown of Shc in control cells, transfected with the empty vector, markedly reduced Decma-induced Erk phosphorylation. This effect could be rescued by the expression of silent mutant p46<sup>Shc</sup> or p52<sup>Shc</sup> but not by silent mutant p66<sup>Shc</sup>. To mediate Erk activation, Shc proteins must be tyrosine phosphorylated on either Tyr239/240 or Tyr313 (Tyr317 in humans). Accordingly, expression of p52<sup>Shc3Y3F</sup> with all the three tyrosines mutated to phenylalanine did not rescue Decma-induced Erk activation (Fig. 3C). Moreover, Decma-induced Erk activation was already reduced by overexpressing p52<sup>Shc3Y3F</sup> and p66<sup>Shc</sup> without the knockdown of endogenous Shc, suggesting that they act in a dominant-negative manner. These results indicate that Decma-induced Erk activation is largely dependent on the p46<sup>Shc</sup> and p52<sup>Shc</sup> proteins.

*Involvement of Src and PI3K in Decma-induced Erk activation* - The E-cadherin adhesion complex is linked to the actin cytoskeleton via catenin proteins. We showed previously that changes in the actin cytoskeleton induce Shc-dependent Erk phosphorylation and uPA upregulation in LLC-PK1 cells (28). Therefore, we examined whether Decma-induced Erk activation requires an intact cytoskeleton. Cytochalasin D (CytD) is a pharmacological agent that caps actin filaments and stimulates ATP hydrolysis on G actin, leading to a very rapid dissolution of the actin cytoskeleton (30). Pretreatment with CytD as well as simultaneous treatment with Decma and CytD at concentrations known to disrupt the cytoskeleton did not prevent Decma-induced Erk phosphorylation in MCF7 cells. CytD treatment alone had no effect on Erk phosphorylation in MCF7 cells but reduced the level in T47D cells. Nevertheless, treatment with Decma resulted in enhanced Erk phosphorylation irrespective of



**Fig 3. Role of Shc in Decma-induced Erk activation.** A, Effect of Decma on Shc phosphorylation and its association with Grb2. After treatment of cells with Decma supernatant for 30 min, 300  $\mu$ g of total cell lysates were immunoprecipitated with anti-Shc antibody and subjected to Western blot analysis. B, Effects of Shc downregulation on Erk activation. Cells were transfected with control (C) or Shc (S) siRNA as described in “Materials and Methods” and treated 3 days later with Decma supernatant or 100 ng/ml TPA as indicated, followed by Western blotting for Shc, phospho-Erk and total Erk levels. C, Rescue by ectopic Shc isoform expression of Erk activation that was suppressed by downregulation of endogenous Shc. Stable cell lines expressing empty vector or silent mutants of HA-p46<sup>shc</sup>, HA-p52<sup>shc</sup>, HA-p52<sup>shc3Y3F</sup>, and HA-p66<sup>shc</sup> were prepared and transfected with siRNA targeting all Shc isoforms (S) or control siRNA (C). After 3 days transfection, cells were treated with Decma supernatant and total cell lysates were analyzed by Western blotting.

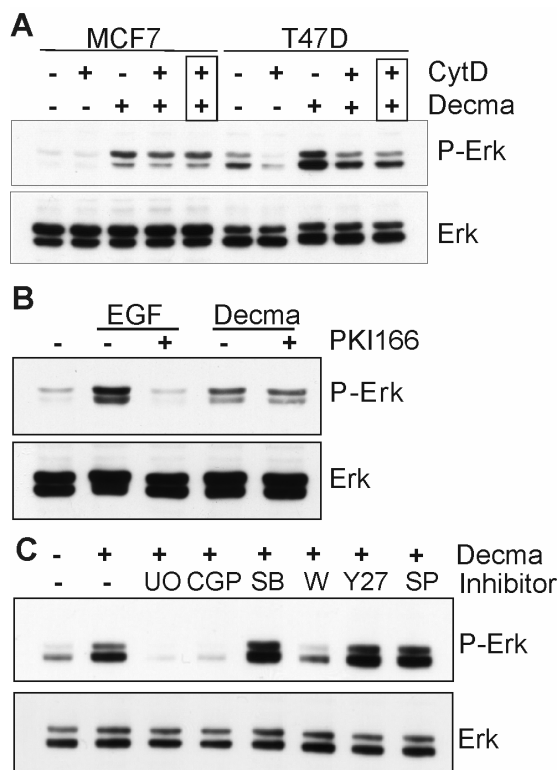
CytD treatment (Fig. 4A). These results suggest that the actin cytoskeleton is not required for Decma-induced Erk activation.

Some reports show a functional cross talk between E-cadherin and the EGFR (14,22,31). To determine whether Decma-induced Erk activation is a result of cross talk between E-cadherin and the EGFR, which might then activate the Shc/Erk pathway, we examined whether EGFR activity was required for Erk activation. As shown in Fig. 4B, Decma-induced Erk phosphorylation was not affected by the EGFR-specific inhibitor PKI166, while EGF-induced Erk activation was completely suppressed, indicating that Decma-induced Erk activation does not rely on transactivation of the EGFR.

In a search for molecules other than Shc lying between E-cadherin and Erk in Decma-induced signaling, we made use of specific inhibitors of various kinases potentially involved in this signaling. Fig. 4C shows that Decma-induced Erk phosphorylation was completely suppressed by the Src-specific inhibitor CGP77675 as well

as by the MEK1 inhibitor UO126 and partially attenuated by the PI3K inhibitor Wortmannin. No effect was observed with inhibitors of p38 MAPK, Rho kinase or JNK, although their activities were confirmed by different control experiments (data not shown). These results show that not only MEK1 and Shc but also Src and PI3K are upstream of Erk in Decma-induced signaling.

**Role of Src in Decma-induced Erk activation** - Since Src kinase activity was found to be necessary for Decma-induced Erk phosphorylation, we examined the activation of Src. Western blot analysis showed that Decma treatment enhanced Src phosphorylation of Tyr416, an indicator of Src activation (Fig. 5A). To assess whether Src is upstream of Shc, Decma-induced Shc tyrosine phosphorylation in the presence of the Src inhibitor CGP77675 was examined. Decma-induced Shc tyrosine phosphorylation and its association with Grb2 were suppressed by the inhibitor, suggesting that Src is located upstream of Shc in this signaling cascade (Fig. 5B). Again, the Rho kinase



**Fig 4. Effect of CytD and several kinase inhibitors on Decma-induced Erk activation.** A, MCF7 and T47D cells were treated separately with Decma supernatant (Decma) for 30 min, with 3  $\mu$ M CytD for 30 min, with 3  $\mu$ M CytD for 45 min followed by Decma for 30 min, or simultaneously with Decma and CytD for 30 min (boxed), and total cell lysates were analyzed by Western blotting for total and phosphorylated Erk levels. B, C, MCF7 cells were pretreated for 45 min with 5  $\mu$ M PKI166 (B), 10  $\mu$ M UO126 (UO), 5  $\mu$ M CGP077675 (CGP), 1  $\mu$ M SB263580 (SB), 100 nM Wortmannin (W), 10  $\mu$ M Y27632 (Y27) or 20  $\mu$ M SP600125 (SP) (C) and then treated with EGF for 10 min (B) or Decma for 30 min (C). Total cell lysates were analyzed as above.

inhibitor Y27634 affected neither Decma-induced Erk activation nor Shc phosphorylation and its association with Grb2 (Fig. 5B). Interestingly, Src inhibition also suppressed the disruption of cell-cell adhesion, the scattered phenotype of the cells and the redistribution of E-cadherin into the cytoplasm (Fig. 5C).

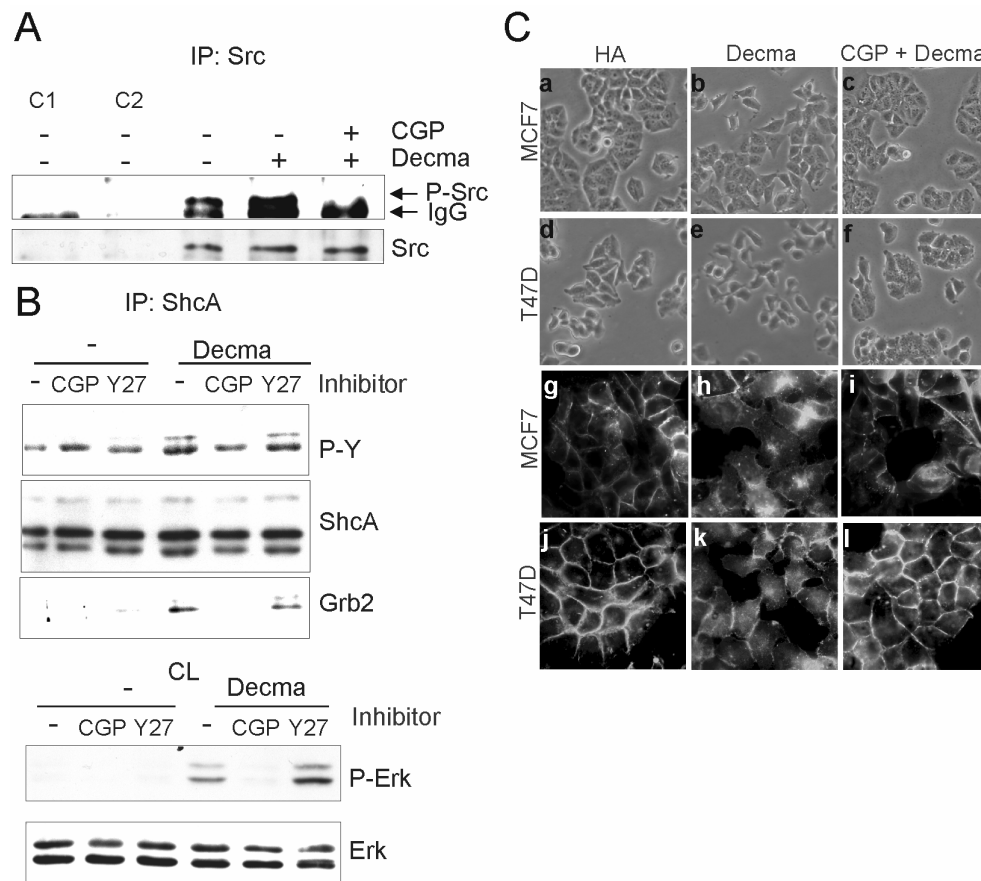
**Role of PI3K in Decma-induced Erk activation** - The partial suppression of the Decma-induced Erk phosphorylation by Wortmannin suggests the involvement of PI3K in this signaling (Fig. 4C). Both Wortmannin and LY29400, two structurally distinct PI3K inhibitors, partially attenuated Erk phosphorylation but completely blocked Decma-induced PKB phosphorylation (Fig. 6A). Interestingly, the Src kinase inhibitor CGP77675

also blocked PKB phosphorylation, suggesting that Src acts upstream of PI3K in Decma-induced signaling. Neither Shc phosphorylation nor its association with Grb2 were affected by Wortmannin (Fig. 6B). Shc knockdown resulted in attenuation of basal PI3K signaling as measured by PKB phosphorylation, but Decma treatment still enhanced PKB phosphorylation (Fig. 6C). Erk phosphorylation was completely suppressed when Shc knockdown and Wortmannin treatment were combined (Fig. 6C). Taken together, these results imply the presence of two parallel pathways downstream of Src leading to Erk activation, one mediated by Shc with a major contribution to Erk activation and the other mediated by PI3K with a minor contribution to Erk activation.

*Decma-induced uPA expression is dependent on Src and PI3K in addition to Erk* - We show here that Src activation is necessary for Erk activation and that PI3K contributes partially. Also, Erk activation is necessary for Decma-induced uPA gene expression (Fig. 2E). As expected, we found that pretreatment with UO126 but also with CGP77675 abolished Decma-induced uPA activation (Fig. 7). Wortmannin, which only partially inhibited Erk activation (Fig. 6A), also reduced uPA gene expression to some extent. These results indicate that blockage of E-cadherin function induces uPA gene expression through signaling pathways involving these kinases.

## DISCUSSION

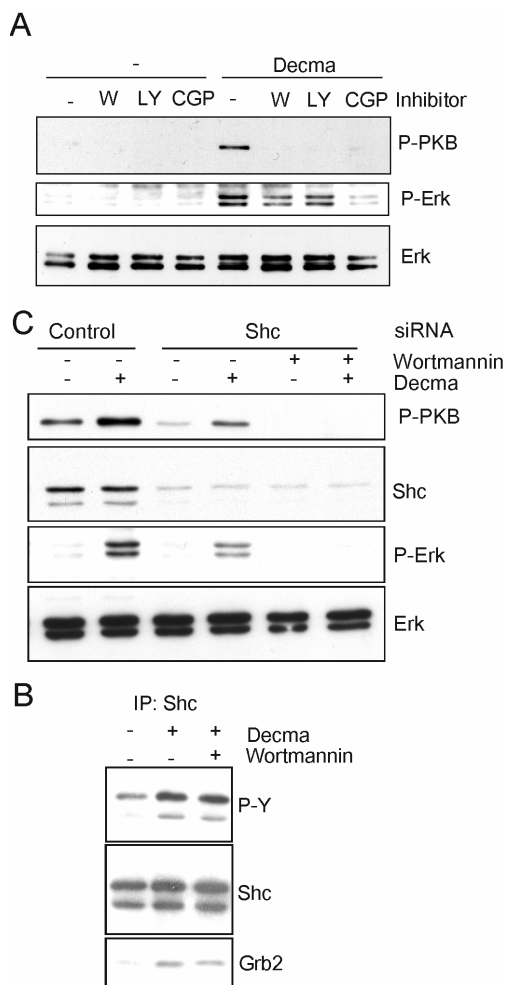
Using the function-blocking antibody Decma, we showed previously that blockage of E-cadherin-mediated cell adhesion results in the upregulation of uPA activation in MCF7 and T47D breast cancer cell lines (25). In this present study, we investigated the underlying molecular mechanisms and showed that disruption of cell-cell adhesion induces Erk signaling downstream of E-cadherin. This Erk activation was Src- and Shc-dependent and resulted in enhanced expression of the uPA gene and, to a lesser extent, of the MMP-9 gene (data not shown). Disruption of cell-cell adhesion by calcium chelation using EGTA has been reported to increase Erk activity (13). Conversely, it was shown that E-cadherin adhesion suppresses basal Erk activity and concomitantly MMP-9 expression (32). It may seem contradictory that Erk is also activated upon re-establishment of



**Fig 5. Involvement of Src in Decma-induced Erk activation.** **A, Activation of Src by Decma.** T47D cells were pretreated with 5  $\mu$ M CGP077675 for 45 min (CGP) as indicated and then treated with Decma supernatant. Total cell lysates (400  $\mu$ g protein) were immunoprecipitated with a polyclonal anti-Src antibody and then subjected to Western blot analysis for Src and phospho-Src (Y416). To discriminate between Src and the heavy chain antibody, the antibody was incubated with only protein A beads and lysis buffer (C1) or the cell lysate was incubated only with protein A beads (C2). **B, Effect of Src inhibitor on Shc and Erk phosphorylation.** T47D cells were pretreated for 45 min with 5  $\mu$ M CGP077675 (CGP) or 10  $\mu$ M Y27632 (Y27) and then treated with Decma supernatant for 30 min. Total lysates (250  $\mu$ g total protein) were immunoprecipitated with anti-Shc antibody and then subjected to Western blot analysis (upper panel). In parallel, the total cell lysates (CL) were examined for Erk and phospho-Erk levels by Western blotting (lower panel). **C, T47D and MCF7 cells** were grown for 2 days to ca. 60% confluence. The cells were then treated for 45 min with 5  $\mu$ M CGP077675 (CGP) (c, f, i, l) and subsequently for 4 h with Decma supernatant (b-c, e-f, h-i, k-l) before recording (a-f) or before immunostaining with the anti-E-cadherin antibody (g-l).

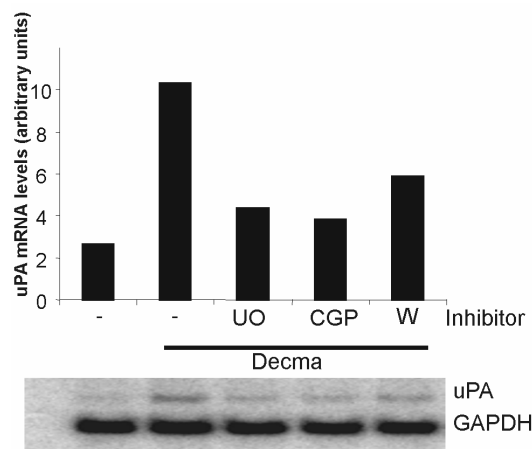
cell-cell adhesion. However, the duration of this activation is much shorter and the underlying molecular mechanisms of the two systems are different. While Erk activation by the establishment of new cell-cell adhesion is transient (5-60 min) and dependent on EGFR (14) (22), Erk activation by the blockage of E-cadherin was sustained (>3 h) and EGFR independent but Src- and Shc-dependent. Interestingly, RNAi-mediated downregulation of E-cadherin reduced Decma-induced Erk activation (Fig. 2D) but did not elevate basal Erk phosphorylation. These results suggest that it is not the absence *per se* of E-cadherin-dependent cell-cell interaction that induces the signaling pathway.

Src has been implicated previously in the control of cell adhesion. Inhibition of Src catalytic activity by overexpression of dominant inhibitory c-Src or by specific inhibitors stabilizes E-cadherin-dependent cell-cell adhesion (33). Conversely, elevated Src activity leads to disorganization of E-cadherin-dependent cell-cell adhesion and cell scattering (34). Fujita *et al.* (35) showed that E-cadherin and  $\beta$ -catenin become ubiquitinated by Hakai upon Src activation, ultimately leading to endocytosis of the E-cadherin complex. Accordingly, in the course of Decma-induced disruption of cell-cell adhesion, Src activation cell scattering and the redistribution



**Fig 6. Role of PI3K in Decma-induced Erk activation.** A, MCF7 cells were pretreated for 45 min with 100 nM Wortmannin (W), 5  $\mu$ M LY294002 (LY), or 5  $\mu$ M CGP077675 (CGP) and then treated with Decma supernatant as indicated. Total cell lysates were subjected to Western blot analysis for phospho-PKB (Ser473), phospho-Erk and total Erk levels. B, MCF7 cells were treated with 100 nM Wortmannin for 45 min and then with Decma supernatant as indicated. Total cell lysates (400  $\mu$ g protein) were immunoprecipitated with anti-Shc antibody and then analyzed for phospho-Shc, total Shc and Grb2 by Western blotting. C, MCF7 cells were transfected with Shc-specific or control siRNA as described in Materials and Methods. After 3 days, transfected cells were pretreated with 100 nM Wortmannin for 45 min and then with Decma supernatant as indicated for 30 min. Levels of phospho-PKB (Ser473), Shc, phospho-Erk and Erk in total cell lysates were determined by Western blotting.

was necessary for the initiation of the signaling of E-cadherin into the cytoplasm. However, the mechanism of Src activation by Decma treatment has not been elucidated. The actin



**Fig 7. Role of Src, PI3K and Erk in Decma-induced uPA upregulation.** MCF7 cells were grown to 60-70% confluency, treated for 45 min with 10  $\mu$ M UO126 (UO), 5  $\mu$ M CGP077675 (CGP) or 100 nM Wortmannin (W), and then with Decma supernatant as indicated. Total RNA (10  $\mu$ g) was subjected to Northern blot analysis (lower panel). The uPA mRNA levels were normalized against GAPDH and presented graphically (upper panel).

cytoskeleton seems not to be necessary for Src activation since CytD failed to prevent Decma-induced Erk activation (Fig. 4A). One possible mechanism of Src activation is through the interaction with p120-catenin. p120-catenin is a Src substrate and has been shown to interact with Src kinase family members (36). This interaction is thought to keep Src kinases in an inactive state. Disruption of E-cadherin-dependent cell-cell adhesion might change the interaction between E-cadherin/p120/Src family members which could allow Src activation. Alternatively, Src activation could be a result of a functional crosstalk between E-cadherin and integrins. Intercommunication between integrins and cadherins has been observed several times (37,38) and Chattopadhyay *et al.* (39) recently reported a complex containing  $\alpha$ 3 $\beta$ 1-integrins and E-cadherin besides other proteins. In an indirect way loss of cell-cell adhesion might generate forces on focal adhesions which could produce integrin-dependent signals. All these possibilities are currently under investigation. Preliminary experiments suggest a functional cross talk between E-cadherin and integrins, given that siRNA-induced knockdown of  $\beta$ 1-integrin reduced Decma-induced Erk phosphorylation (unpublished data).

Using siRNA against all isoforms of Shc, we found this adaptor protein to be essential for Decma-induced Erk activation. Moreover,

expression of silent mutants of Shc isoforms showed that only p46<sup>Shc</sup> and p52<sup>Shc</sup> rescued the effect of the siRNA. Overexpression of p66<sup>Shc</sup> not only failed to rescue Erk activation, but in fact had a negative effect comparable to the effect of overexpressed dominant negative p52<sup>Shc3Y3F</sup>. These results support previous observations that p66<sup>Shc</sup> is a negative regulator of EGF-induced Erk activation and *c-fos* promoter activation (21). In agreement with this, we showed recently that p66<sup>Shc</sup> is unable to rescue cytoskeleton reorganization-induced Erk activation after siRNA mediated knockdown of all isoforms of endogenous Shc (28).

Decma-induced Shc tyrosine phosphorylation and its binding to Grb2 were completely repressed by pretreatment with a Src inhibitor, suggesting that Src acts upstream of Shc in Decma-induced signaling. In accordance with this observation, *in vitro* kinase assays have demonstrated that Src is able to phosphorylate all three tyrosine residues of Shc proteins directly (40) and is responsible for Shc phosphorylation upon fibronectin (41) and PDGF stimulation (42). FAK has been reported to form a complex with Shc and Grb2 upon CytD treatment in LLC-PK1 cells (28) and upon fibronectin stimulation in NIH3T3 fibroblasts (41). However, it is unlikely that FAK plays a role in Decma-induced Erk activation. No interaction of Shc and FAK was detected upon Decma treatment and overexpression of dominant-negative FRNK failed to abrogate Erk activation (data not shown).

While the Src/Shc/Erk pathway plays a major role in Decma-induced Erk activation, Decma also induced the Src/PI3K/Erk pathway. Treatment of MCF7 or T47D cells with Wortmannin partially reduced Erk activation without affecting Shc phosphorylation. RNAi-mediated knockdown of Shc reduced the basal activity of the PI3K pathway as measured by PKB phosphorylation. Nevertheless, Decma treatment still enhanced PKB phosphorylation, indicating a Shc-independent pathway for Decma-induced PKB activation. Effects of Wortmannin on Erk have been reported in several cell systems: in T lymphocytes (43), Cos 7 cells (44), and a CHO-derived cell line (45). However, the site at which the PI3K signaling feeds into the Erk activating pathway varies in these systems: at the Ras, Raf or MEK activation level. In the Decma-induced pathway, the signal from PI3K could

contribute to Erk activation by acting at any of these sites, except upstream of Shc.

We disrupted cell-cell adhesion by physical means using the function-blocking antibody Decma in order to reproduce a process observed in some types of tumorigenesis. During the course of tumor progression, the ectodomain of E-cadherin can be detached by matrilysin and stomilysin-1, releasing an 80-kDA soluble E-cadherin fragment (sE-cad)(10). sE-cad has been found in urine and serum of cancer patients and correlates with a poor prognosis (46-48). In tissue culture, it induces scattering of epithelial cells (49), inhibition of E-cadherin-dependent cell aggregation and invasion of cells into type I collagen (10). Furthermore, sE-cad stimulates the upregulation of MMP-2, MMP-9 and MTI-MMP expression in human lung tumor cells, as reported by Noe and colleagues (10). To explain all these effects, the authors suggested the presence of a signal transduction pathway induced either directly by sE-cad or indirectly by the disruption of cell-cell contact (50). It may be argued that the signaling described in this report is a consequence of Decma acting as a ligand for E-cadherin. However, several lines of evidence suggest it is the disruption of cell-cell adhesion that is attributable for Decma-induced signaling activation. First, a second antibody against the extracellular domain of E-cadherin which did not disrupt cell-cell adhesion did not induce Erk activation. Second, inhibition of Src blocked the disruption of cell-cell adhesion and at the same time prevented Erk activation. Third, Decma recognizes an epitope located close to the membrane proximal part of the extracellular domain of E-cadherin (51). Structural changes in this membrane proximal region has been shown to change the adhesive properties of cells. Finally, Decma and sE-cad share common features that they disrupt cell-cell adhesion and induce signaling despite the fact that they interact with E-cadherin in different manners. Therefore, it is most likely that it is the disruption of E-cadherin-mediated cell-cell adhesion that triggers a signal transduction pathway leading to Erk activation and uPA gene expression.

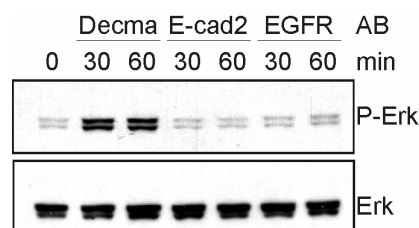
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**Supplementary Fig.** MCF7 cells were treated with 50  $\mu$ g/ml of the indicated antibody for the indicated time. Total cell lysates were subjected to Western blot analysis for phospho-Erk and total Erk levels.

1

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## 2.5 Supplementary data to 2.4

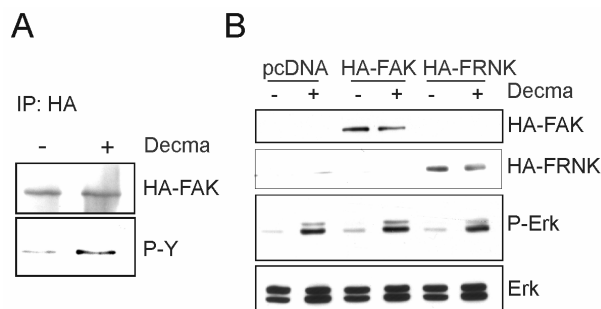
### 2.5.1 Role of FAK in Decma-induced Erk activation

We have shown that Src activity is necessary for Decma-induced Erk activation. The Focal Adhesion Kinase (FAK) is not only one of the major interacting partners of Src, but it can also activate Erk signaling through its interaction with Shc and Grb2 (Schlaepfer et al., 1999). We, therefore, asked whether FAK is activated upon Decma treatment, and found that its tyrosine phosphorylation is enhanced compared with the basal level (Fig. 2.5.1A). To further investigate whether FAK participates in Decma-induced Erk activation, the effect of overexpressed FRNK, a dominant-negative FAK mutant (Richardson and Parsons, 1996), was examined. FRNK expression had no effect on Erk phosphorylation, making

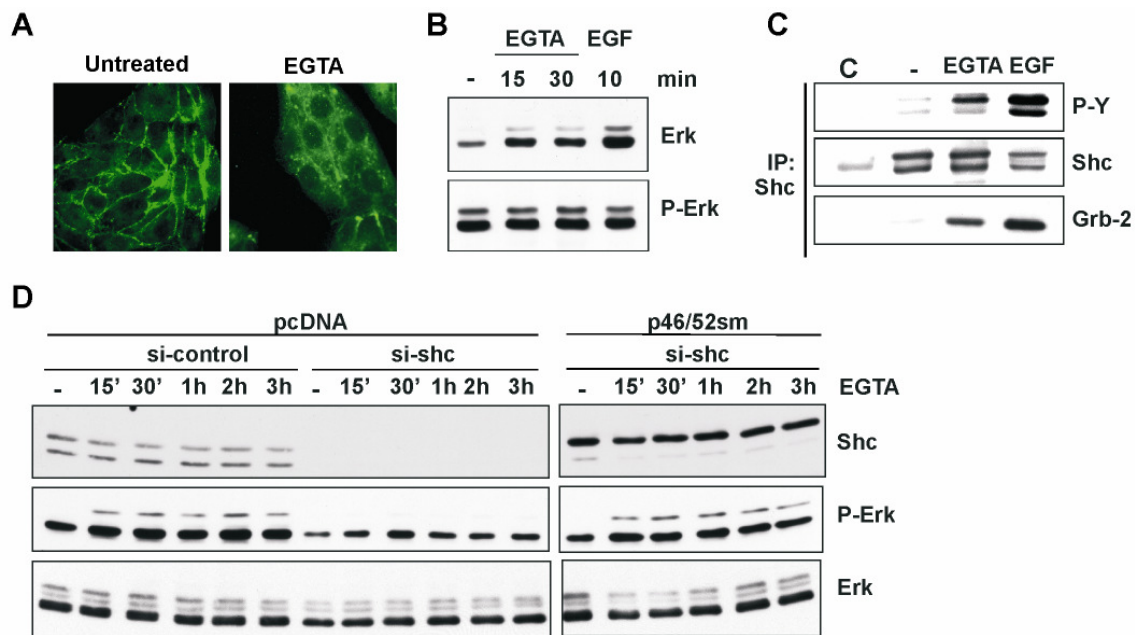
a role for FAK in coupling Src activation to the Erk signaling pathway in this system unlikely (Fig. 2.5.1B). The functionality of the construct has been shown in another study from our laboratory (Irigoyen and Nagamine, 1999).

### 2.5.2 Disruption of cell-cell adhesion using EGTA in LLC-PK1 cells

In the non-transformed pig epithelial cell line LLC-PK1, the E-cadherin function-blocking antibody Decma does not disrupt cell-cell adhesion. However, E-cadherin-dependent cell-cell adhesion is calcium-dependent and can therefore be disrupted using calcium-chelating agents such as EGTA. As illustrated in Fig. 2.5.2A, almost all E-cadherin protein that is normally localized at sites of cell-cell adhesion was internalized into the cytoplasm 30 min after EGTA treatment. Similar to Decma treatment in MCF7 and T47D cells, EGTA treatment caused Erk activation, Shc phosphorylation, and Shc association with Grb2 (Fig. 2.5.2B, C). Knockdown of Shc using siRNA prevented EGTA-induced Erk phosphorylation, suggesting that Erk activation is dependent on Shc. Moreover, expression of HA-p46<sup>Shcsm</sup> and HA-p52<sup>Shcsm</sup>, which escaped targeting of the siRNA (knockdown-in), rescued sustained Erk phosphorylation induced by EGTA (Fig. 2.5.2D). In conclusion, EGTA disrupts cell-cell adhesion and induces Shc-dependent sustained Erk activation in a manner, which is reminiscent of the signaling induced by disruption of cell-cell adhesion using Decma in MCF7 and T47D cells.



**Figure 2.5.1: Role of FAK in Decma-induced Erk activation.** T47D cells were transiently transfected with pcDNA, HA-FAK, or HA-FRNK and then treated with Decma supernatant. Total cell lysates were immunoprecipitated with HA antibody (A) or directly subjected to Western blot analysis (B).

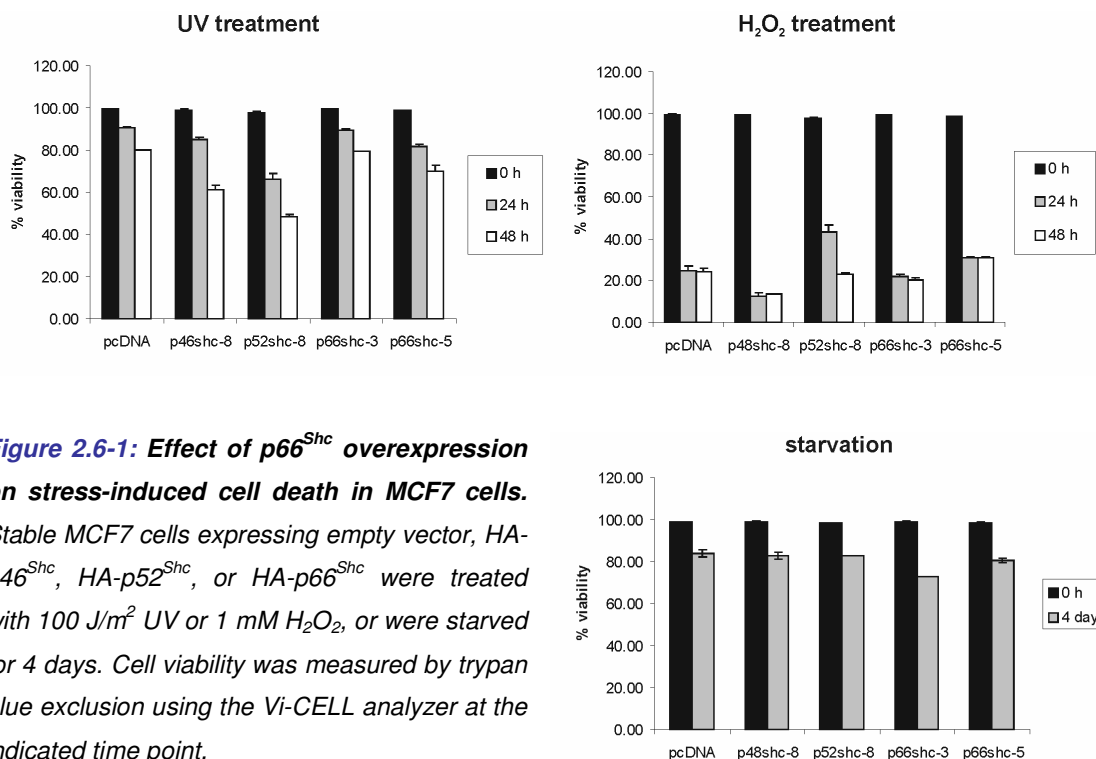


**Figure 2.5.2: A. Effect of EGTA on LLC-PK1 cells.** LLC-PK1 cells were treated for 30 min with 4 mM EGTA and 1 mM  $MgCl_2$ , and then immunostained with anti-E-cadherin antibody. **B.** LLC-PK1 cells were treated with 4 mM EGTA/1 mM  $MgCl_2$  or 50 ng/ml EGF for the indicated time, and total cell lysate was subjected to Western blot analysis. **C.** After treatment of cells with 4 mM EGTA/1 mM  $MgCl_2$  for 15 min, 300  $\mu$ g of total cell lysates were immunoprecipitated with anti-Shc antibody and subjected to Western blot analysis. **D.** Cell lines stably expressing empty vector or HA-p46/52<sup>Shcsm</sup> were transfected with control or all-shc siRNA. Three days later, cells were treated with 4 mM EGTA/1 mM  $MgCl_2$  for the indicated time and total cell lysate was analyzed by Western blotting.

## 2.6 Role of p66<sup>Shc</sup> in regulating cell survival in epithelial cells

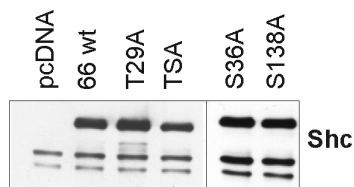
p66<sup>Shc</sup> is implicated in the regulation of cell survival in response to oxidative stress, and the absence of p66<sup>Shc</sup> renders cells more resistant to UV-, H<sub>2</sub>O<sub>2</sub>-, or starvation-induced cell death (1.1.2.7) (Migliaccio et al., 1999; Nemoto and Finkel, 2002). This function of p66<sup>Shc</sup> has been attributed to the phosphorylation of the serine residue S36. Still, the precise mechanism underlying this role is not fully understood. In addition, it is not clear whether this is only restricted to certain cell types, such as fibroblasts, endothelial cells, and T-cells, or whether this represents a general mechanism. To investigate the role of p66<sup>Shc</sup> in regulating the survival of epithelial cells, p66<sup>Shc</sup>-expressing and non-expressing

MCF7 cells (Fig. 2.3-2) were analyzed. Starvation, UV and H<sub>2</sub>O<sub>2</sub> treatment of MCF7 cells resulted in loss of cell viability as measured by trypan blue exclusion. However, no p66<sup>Shc</sup>-dependent change in cell viability was observed after any of these treatments (Fig 2.6-1). The same experiments were repeated with LLC-PK1 epithelial cells. Similarly to MCF7 cells, wild-type LLC-PK1 cells do not express p66<sup>Shc</sup>. Again, p66<sup>Shc</sup>-overexpressing cell lines were generated. Because the effect of p66<sup>Shc</sup> on stress-induced cell death was reported to be dependent on serine 36, cells overexpressing p66<sup>Shc</sup> carrying a serine to alanine mutation (S36A) at this site were made. Since p66<sup>Shc</sup> contains an additional threonine phosphorylation site with as yet unknown function, a mutation was introduced at this site too (T29A). Finally, cell lines overexpressing p66<sup>Shc</sup> containing serine



**Figure 2.6-1: Effect of p66<sup>Shc</sup> overexpression on stress-induced cell death in MCF7 cells.** Stable MCF7 cells expressing empty vector, HA-p46<sup>Shc</sup>, HA-p52<sup>Shc</sup>, or HA-p66<sup>Shc</sup> were treated with 100 J/m<sup>2</sup> UV or 1 mM H<sub>2</sub>O<sub>2</sub>, or were starved for 4 days. Cell viability was measured by trypan blue exclusion using the Vi-CELL analyzer at the indicated time point.

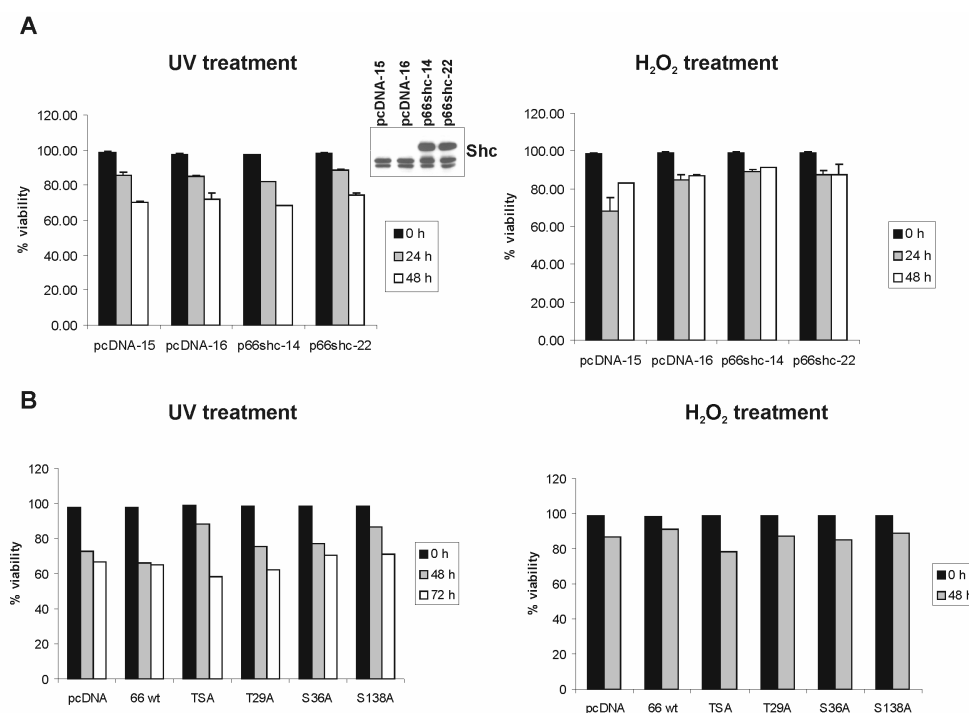
36/threonine 29 double mutation (TSA) were generated and, as control, cell clones expressing p66<sup>Shc</sup> mutated at serine 138 (S138A), which is implicated in PTP-PEST binding (Fig 2.6-2).



**Figure 2.6-2: LLC-PK1 cells stably overexpressing p66<sup>Shc</sup>.** LLC-PK1 cells were stably transfected to overexpress wild-type HA-p66<sup>Shc</sup> and HA-p66<sup>Shc</sup> threonine or serine to alanine mutants. The depicted clones were used for further experiments.

LLC-PK1 cells were more resistant to all stress-inducing agents, and higher doses were necessary to induce cell death. Again, overexpression of p66<sup>Shc</sup> did not render these cells more sensitive to UV- or H<sub>2</sub>O<sub>2</sub>-induced cell death (Fig 2.6-3A). In addition, no changes in cell viability were observed when either of the p66<sup>Shc</sup> phosphorylation mutants was overexpressed in these cells (Fig 2.6-3B).

Various different experimental conditions (dosage of stress, duration after stress, starvation) were used, but we were unable to see p66<sup>Shc</sup>-dependent effects on cell viability in neither MCF7 nor LLC-PK1 cells, suggesting that p66<sup>Shc</sup> might not be involved in regulating cell survival upon stress in epithelial cells.



**Figure 2.6-3: Effect of p66<sup>Shc</sup> overexpression on stress-induced cell death in LLC-PK1 cells.** **A.** Two clones of stable LLC-PK1 cells expressing empty vector or HA-p66<sup>Shc</sup> were treated with 200 J/m<sup>2</sup> UV or 1 mM H<sub>2</sub>O<sub>2</sub>. **B.** Stable LLC-PK1 cells expressing empty vector, HA-p66<sup>Shc</sup> wild-type, HA-p66<sup>ShcTSA</sup>, HA-p66<sup>ShcT29A</sup>, HA-p66<sup>ShcS36A</sup>, or HA-p66<sup>ShcS138A</sup> were treated with 200 J/m<sup>2</sup> UV or 1.5 mM H<sub>2</sub>O<sub>2</sub>. Cell viability was measured by trypan blue exclusion using the Vi-CELL analyzer at the indicated timepoints.

### 3. Discussion

#### 3.1 Isoform-specific knockdown and knockdown-in of Shc using siRNA

We have generated a system which allows downregulation and expression of single Shc isoforms in a short time period in tissue culture. This system should be applicable and functional for the study of almost all genes which are expressed as multiple isoforms. Moreover, the knockdown-in system can be useful for validating specificity or mutational analysis in follow-up experiments in which the target gene is restored by vector-based expression of a wild-type or mutated form of the gene. In our studies, coding sequences were targeted by siRNA. Therefore, the expression of siRNA-resistant proteins required point mutations in the region corresponding to the siRNA. Alternatively, 3' or 5' UTR regions could be targeted ([http://www.ambion.com/techlib/posters/RNAi\\_0302.html](http://www.ambion.com/techlib/posters/RNAi_0302.html)) (Elbashir et al., 2002; Tsuda et al., 2005). Targeting UTR sequences facilitates knockdown-in experiments because under these conditions it is unnecessary to introduce silent mutations in the vector-encoded genes. However, since UTR sequences are known sites of mRNA-binding proteins, some researches prefer to avoid these regions (Dykxhoorn et al., 2003; Elbashir et al., 2002).

Although siRNAs are thought to act in a very specific manner, we observed side effects when siRNAs were used at high concentrations (100 nM). Nonspecific events were also noticed by other laboratories when siRNA concentrations of 100 nM or higher were used in mammalian cells. A comparison of expression profiles resulting from silencing of the same target gene by different siRNAs

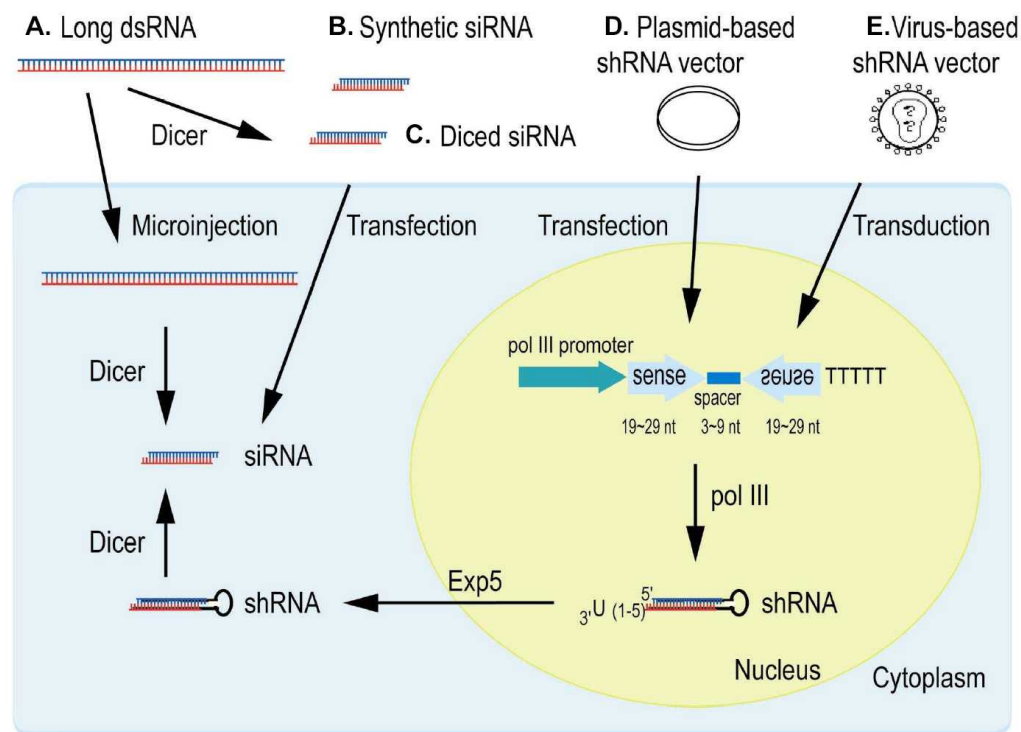
revealed siRNA-specific, rather than target-specific, signatures including silencing of non-targeted genes (Jackson et al., 2003). Another study has shown that transfection of siRNA at concentrations of 100 nM induced a significant number of genes, many of which are known to be involved in apoptosis and stress response (Semizarov et al., 2003). Reduction of the siRNA concentration to 20 nM eliminated this nonspecific response. Taking our observation and these reports into account, a number of different controls for siRNA experiments are recommended. In line with this, a recent editorial in *Nature Cell Biology* (2003) published a list of control experiments required for siRNA experiments. These include: (i) minimization of siRNA concentration, (ii) use of scrambled siRNA control, (iii) use of multiple siRNAs for a single target, (iv) examination of downregulation of mRNA and protein levels, and (v) rescue experiments as ultimate and best control. If a careful design of siRNAs, similar to those published by Elbashir *et al.* (Elbashir et al., 2002) and Semizarov *et al.* (Semizarov et al., 2003), is combined with the suggested controls, siRNAs are a highly specific tool for targeted gene knockdown.

In contrast to fungi, plants, and worms, which can replicate siRNA, there is no indication of siRNA amplification in mammals (Dykxhoorn et al., 2003). Likewise, we and others have shown the transient nature of siRNA-directed silencing by transfection into mammalian cells (Dykxhoorn et al., 2003). To prolong siRNA-induced knockdown, we suggested repeated transfection of siRNAs into cells. Later, several reports appeared which described vector-based siRNA expression systems (Amarzguioui et al., 2005;

Brummelkamp et al., 2002; Miyagishi and Taira, 2002; Yu et al., 2002). These allow stable expression of siRNA and can be designed in an inducible manner. Today, several strategies have been developed for RNAi in mammalian cells: they are summarized in Fig 3.1 (Kim, 2005).

The study describing the isoform-specific knockdown and the knockdown-in was published at the beginning of 2002, when the mechanisms underlying siRNA-directed

silencing were not fully understood. In this regard, our report not only offered a useful application of siRNA for the investigation of proteins expressed in multiple isoforms, but also gave some insights into the mechanisms of RNAi. These are discussed in section 2.1. Today, the mechanisms underlying RNAi have been well investigated (section 1.3). The use of RNAi has been further developed and it has become an effective, widely used method for the analysis of gene function.



**Figure 3.1: Various strategies for RNAi in mammalian cells.** **A.** Long dsRNAs can induce specific RNAi in oocytes, early embryos, and undifferentiated embryonic stem cells. **B.** Chemically synthesized siRNA duplexes can be efficiently transfected into cultured cells. **C.** siRNA can be prepared in vitro from dsRNAs by incubating with recombinant Dicer protein. The diced products are purified based on their size (~21 nt) and transfected into cells. **D.** Short hairpin RNAs (shRNAs) are expressed in the nucleus from expression plasmids: the RNA polymerase (pol) III-derived expression system is shown here as an example. Upon export by Exportin 5 (Exp5), shRNAs are processed by Dicer-releasing siRNAs. **E.** ShRNA expression cassette can be delivered by viral vectors such as retroviral vector, lentiviral vector, and adenoviral vector (taken from (Dykxhoorn et al., 2003)).



## 3.2 Role of Shc in mediating Erk activation

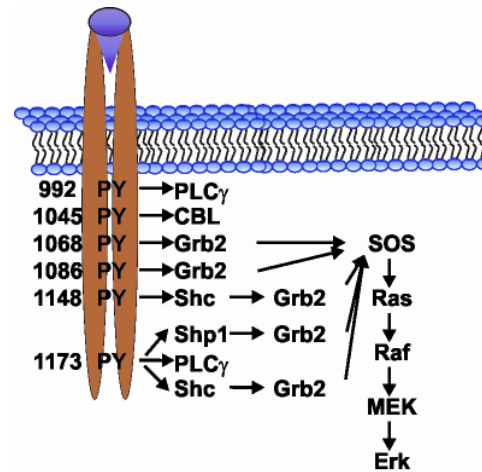
### 3.2.1 Shc is dispensable for EGF-induced Erk activation

Shc has been found as an adaptor protein that recruits the Grb2/SOS complex to the membrane in order to activate Ras, thereby coupling activated RTKs, such as EGFR, to Erk activation (Ravichandran, 2001). Through this action, Shc is involved in regulating the proliferation of mammalian cells (Pelicci et al., 1992). In an attempt to study the isoform-specific contribution of Shc proteins to EGF-induced signaling, we applied the siRNA-directed knockdown technique to several epithelial cell lines. Isoform-specific knockdown and knockdown of all Shc proteins did not influence EGF-induced Erk activation or response. Therefore, the results argue that EGF signaling is not dependent on Shc proteins in epithelial cells. Interestingly, Shc knockdown had also no effect on NGF-induced Erk activation in PC12 cells (data not shown). However, further experiments are required to investigate whether the absence of Shc has more subtle effects on EGF signaling. Shc acts upstream of Ras in EGFR signaling. We limited our investigation on Erk phosphorylation to the classical output of Ras activation. However, there are several lines of evidence suggesting that PI3K is another Ras-effector molecule (Rommel and Hafen, 1998). Whereas stimulation of the Ras/Raf/Erk pathway is seen at very low concentrations of growth factors, PI3K activation is only observed at higher concentrations (Pawson and Saxton, 1999). Therefore, it can be argued that Shc knockdown reduces the extent of Ras

activation which would still lead to the activation of the Raf/Erk pathway but would reduce Ras-mediated activation of PI3K. However, it is not clear whether this would have any physiological role, because PI3K is directly activated by EGFR. In a different cellular system, Hashimoto *et al.* (Hashimoto et al., 1999) have shown that Shc is not necessary for Ras activation by EGFR, but is important for JNK activation. It remains to be investigated whether JNK activation is changed upon Shc knockdown in epithelial cells. p66<sup>Shc</sup> has been proposed as a negative regulator of EGF-induced Erk activation. Isoform-specific knockdown of p66<sup>Shc</sup> did not change EGF-induced Erk activation in HeLa, PNT2, or PC3 epithelial cells. Conversely, no change in EGF-mediated cell viability or proliferation was observed when p66<sup>Shc</sup> was overexpressed. However, the experiments were done using 50 ng/ml EGF. Further experiments will show whether p66<sup>Shc</sup> influences these parameters at low EGF concentrations.

Ignoring these cautions for a moment, the data represented here seem contradictory to the current established role of Shc proteins in cell signaling. However, reports which demonstrate that Shc is essential in mediating Erk activation downstream of EGFR are based on studies in which dominant negative Shc mutants were overexpressed (Gotoh et al., 1995; Gotoh et al., 1997). It must be noted that the binding of Grb2 to EGFR, similar to most of the RTK, is redundant. Grb2 can be associated with the receptor either directly via its SH2 domain or indirectly via binding to EGFR-associated Shc and, most likely, Shp1 (Src homology phosphatase) (Chen et al., 1996; Hynes and Lane, 2005; Minoo et al., 2004) (Fig 3.2.1-1). Domain-mediated protein

interactions are dependent on both the affinity and the relative concentration of the binding partner: high affinity interaction would be favored at low concentrations of the target molecule, but could be displaced by low affinity interactions driven by high concentrations of affinity partners. Therefore, it is possible that overexpression of dominant negative Shc prevents the association of the receptor not only with endogenous Shc, but also with other proteins, such as Grb2, resulting in the inhibition of Erk activation. In contrast, the removal of Shc by siRNA would not interfere with the association of Grb2 or Shp1 with the receptor. Redundancy of adaptor proteins was also found in *Drosophila*. DShc, DRK (Grb2 in mammals) and DOS (daughter of sevenless) have been shown to act in parallel to transduce signals from the RTK torso (Luschig et al., 2000). Therefore, loss-of-function mutations in *dshc* affected RTK function only partially. Moreover, dShc seems to act in signaling of only a subset of RTKs, indicating that dShc confers specificity to receptor signaling. In mammals, Shc is phosphorylated downstream of all RTKs known to date (Luzi et al., 2000). However, the physiological relevance of Shc phosphorylation might depend on the repertoire of other adaptor proteins expressed at the same time. The relative contribution of Shc to signaling of each of the receptors should be addressed using siRNA-mediated knockdown, and not overexpression dominant negative Shc. The existence of interactions between Shc and Grb2 with EGFR, with each other, and with a subset of cellular proteins raises the question of how interactions are controlled. Do all possible interactions occur in a single cell and, if so, does activation of one pathway influence activation of alternative pathways?



**Figure 3.2.1-1: Schematic representation of the main autophosphorylation sites in EGFR and of signaling molecules associated with these sites.** Each receptor chain becomes phosphorylated on multiple sites and binds specific SH2-containing proteins. The receptor has redundant interactions, for example with Grb2. This figure is illustrative and not comprehensive: some binding partners and pathways, including PI3K, Src, and STATS, are not depicted.

As already mentioned above, protein interactions are influenced by their binding affinity to each other and by their relative concentrations. It is also important to recognize that the “local” abundance of a protein may determine its availability for binding: co-localization will favor interaction even when affinity is low. Protein association patterns can vary within the same cell depending on the stimulus and timing of the stimulation. Preliminary data suggest that Shc proteins might play a role in stress-induced activation of MAPKs. In LLC-PK1 cells, knockdown of Shc did not alter Erk activation induced by EGF and TPA (Fig 3.2.1-2 lanes 1-6). However, Erk activation was slightly reduced after H<sub>2</sub>O<sub>2</sub> and UV treatment in the absence of Shc (Fig 3.2.1-2 lanes 7-10).

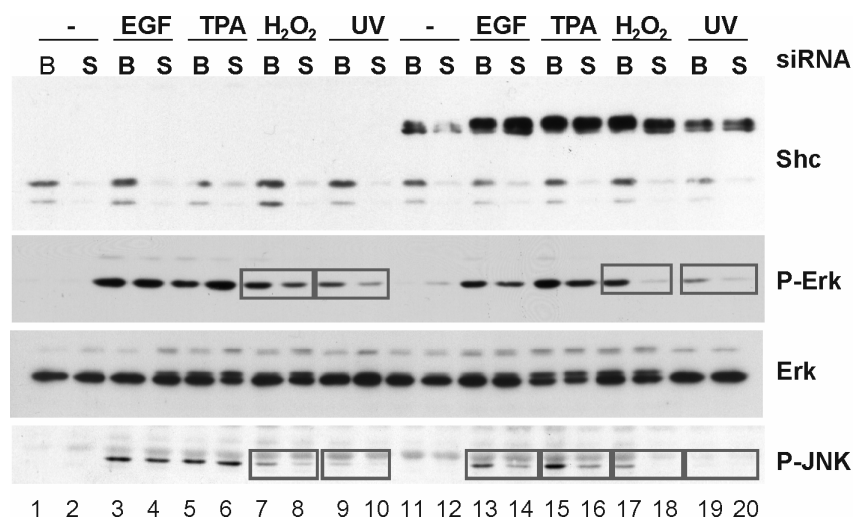
Interestingly, only expression of p66<sup>Shc</sup> (knockdown-in) enhanced the reduction of H<sub>2</sub>O<sub>2</sub>- and UV-induced Erk activation seen before (Fig 3.2.1-2 lanes 17-20). Similar effects were seen for JNK activation (Fig 3.2.1-2 lane 13-18). These results indicate that Erk and JNK activation stimulated by stress, such as UV and H<sub>2</sub>O<sub>2</sub>, are mediated, at least partially, by Shc proteins and that p66<sup>Shc</sup> is able to modulate this MAPK activation.

Interactions between various proteins are also controlled in a tissue-dependent manner. Different cell types may favor different protein associations. Shc is most highly expressed in adipocytes, smooth muscle cells, and cardiac myocytes. The Shc knockout embryo died because of defects in heart development and the cardiovascular system. Therefore, Shc is expected to play a more important role in these tissues, and future experiments to deepen our understanding of the underlying molecular

mechanisms of Shc should target these tissues.

### 3.2.2 Shc mediates Erk activation downstream of E-cadherin

Loss of Shc affects fibroectin-induced Erk activity, focal complex distribution, the actin cytoskeleton, and cell-cell contacts (Lai and Pawson, 2000). In addition, Wary *et al* (Wary *et al.*, 1996) demonstrated that Shc is necessary for integrin-induced Erk activation, and we have shown that Shc plays an essential role in CSR-induced Erk activation (Faisal *et al.*, 2004). Taken together, these results suggest that Shc might be important for Erk activation mediated by non-receptor tyrosine kinases. We have recently shown that uPA secretion is induced upon disruption of

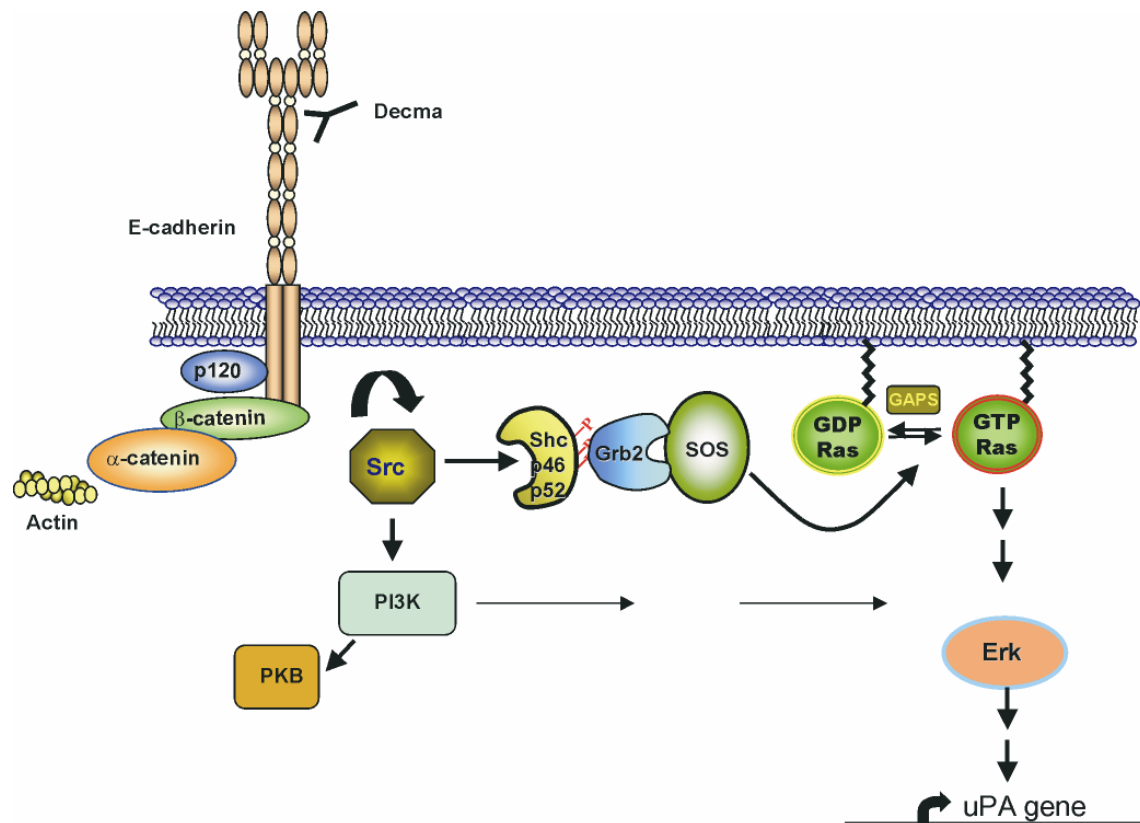


**Figure 3.2.1-2: Effect of Shc knockdown and p66<sup>Shc</sup> overexpression on MAPK activation.** LLC-PK1 cells expressing either empty vector or p66<sup>Shc</sup> were transfected with buffer (B) or si-shc1 (S) and three days later treated with 50 ng/ml EGF, 100 nM TPA, 1 mM H<sub>2</sub>O<sub>2</sub>, or 200 J/m<sup>2</sup> UV. Total cell lysate was analyzed by Western blotting. Boxes indicate changes in the phosphorylation level of Erk or JNK in dependence on Shc proteins.

cell-cell adhesion (Frixen and Nagamine, 1993). Knowing that uPA gene expression is often regulated through stimulation of the MAPK pathway (Besser et al., 1995a; Besser et al., 1995b; Irigoyen and Nagamine, 1999) the question was raised of whether disruption of cell-cell adhesion induces MAPK activation, and whether Shc would play a role in mediating this MAPK activation.

In section 2.3 we describe and discuss the fact that disruption of E-cadherin-dependent cell-cell adhesion does indeed stimulate a previously unknown signaling pathway (Fig 3.4.2). This pathway directly links disruption of

E-cadherin-dependent cell-cell adhesion to the induction of the uPA gene, two events which are strongly involved in metastasis. Upon disruption of cell-cell adhesion through the function-blocking antibody Decma, Src becomes activated and induces Shc/Grb2 association leading to the activation of Erk and finally to uPA gene expression. On the other hand, Src mediates PI3K and PKB activation. PI3K activation contributes partially to Erk activation.



**Figure 3.2.2: Schematic representation of the signaling pathway induced by disruption of E-cadherin-dependent cell-cell adhesion.** Perturbation of E-cadherin-dependent cell-cell adhesion leads to Src activation, followed by p46/p52<sup>Shc</sup> phosphorylation, which mediates Erk activation through Ras/Raf/MEK1. This results in enhanced uPA gene expression. Active Src also mediates PI3K/PKB activation. PI3K signaling contributes to Erk activation.

Proteolytic cleavage of E-cadherin by MMPs, such as ADAM10, has been shown to induce translocation of  $\beta$ -catenin into the nucleus and increase the expression of the  $\beta$ -catenin downstream gene cyclin D1 (Maretzky et al., 2005). uPA has also been described as a target of  $\beta$ -catenin (Hiendlmeyer et al., 2004). Therefore, it would be interesting to see whether Decma-induced uPA gene expression is partially mediated by  $\beta$ -catenin. It is worth noting that the same amount of  $\beta$ -catenin co-immunoprecipitated with E-cadherin at least 30 min after Decma treatment (data not shown). Similarly, using immunostaining we could not detect nuclear  $\beta$ -catenin localization upon Decma treatment (data not shown). However, we did not perform a promoter activation assay to see whether TCF/LEF-dependent transcription becomes activated upon disruption of cell-cell adhesion through Decma.

Disruption of cell-cell adhesion using EGTA also induced Shc-dependent Erk activation in LLC-PK1 cells (section 2.5.2 Fig. 2.5.2), indicating that the signaling pathway described earlier is not restricted to Decma-induced disruption of cell-cell adhesion. Interestingly, a recent report shows that disruption of cell-cell adhesion using EGTA leads to the activation of the small GTPase Rap1, a crucial regulator of inside-out activation of integrins (Balzac et al., 2005). The authors also observed an increase in Src activity, which was required for Rap1 activation. Further investigation revealed that E-cadherin endocytosis is necessary for Rap1 activation. Src is a major player in regulating endocytosis of E-cadherin (Frame, 2002; Fujita et al., 2002; Palovuori et al., 2003). Treatment of the Src inhibitor prevented disruption of cell-cell adhesion and internalization of E-cadherin upon treatment with Decma. Therefore, it would be interesting to see whether Decma-

induced Erk activation is dependent on E-cadherin endocytosis. However, CytD treatment, which is believed to block E-cadherin internalization through disruption of the actin cytoskeleton, did not inhibit Erk activity, arguing against this possibility (section 2.3 Fig 4). To find a conclusive answer, experiments using specific pharmacological inhibitors of endocytosis have to be done.

In this study, we clearly showed that Decma-induced Erk activation can be mediated by p46/p52<sup>Shc</sup> but not by p66<sup>Shc</sup>. Moreover, overexpression of p66<sup>Shc</sup> led to a dominant negative pattern. The reason for p66<sup>Shc</sup> being unable to rescue Decma-induced Erk activation is not understood. In a different study, we have shown that p46/p52<sup>Shc</sup>, but not p66<sup>Shc</sup>, rescued CSR-induced Erk activation in LLC-PK1 cells (Faisal et al., 2004). Investigation of the p66<sup>Shc</sup> phosphorylation status revealed that p66<sup>Shc</sup> was not phosphorylated at its tyrosine residues upon CSR. On the other hand, overexpression of p66<sup>Shc</sup> had no dominant negative effect on CSR-induced Erk activation. We have not investigated yet whether p66<sup>Shc</sup> becomes tyrosine-phosphorylated upon disruption of cell-cell adhesion. The fact that its overexpression interfered with Decma-induced Erk activation argues for an active role of p66<sup>Shc</sup> in this signaling. A mechanism where p66<sup>Shc</sup> competes with the other isoforms for Grb2 binding, or where p66<sup>Shc</sup> binds to RasGAP to downregulate Ras activation as described in section 1.1.2.6 (Fig 1.1.2.6-1), can be envisioned. More experiments will be needed to test this hypothesis. However, the action of p66<sup>Shc</sup> seems to be cell type- and/or stimulus-dependent.

Taken together, we found that Shc is not essential for growth-factor-induced Erk activation. In contrast, we found a novel pathway downstream of E-cadherin in which p46/p52<sup>Shc</sup> are required to mediate Erk activation. In context with other studies showing that the absence of Shc impairs focal complex distribution, the actin cytoskeleton, and cell-cell contacts (Lai and Pawson, 2000), as well as that Shc is essential for CSR- and integrin-induced Erk activation (Faisal et al., 2004; Wary et al., 1996), we propose that Shc proteins play a more important role in mediating growth factor-independent Erk activation which involves the action of soluble tyrosine kinases. This hypothesis is also supported by *in vivo* studies demonstrating that Shc is essential for signaling downstream of the pre-TCR that uses non-receptor tyrosine kinases to phosphorylate Shc proteins (Pacini et al., 1998; Zhang et al., 2002).

### 3.3 The role of p66<sup>Shc</sup> in stress response

The absence of p66<sup>Shc</sup> confers resistance to oxidative stress in mouse embryo fibroblasts, endothelial cells, and T-cells (section 1.1.2.7). We wanted to use two epithelial cell lines to study p66<sup>Shc</sup> function and the role of its serine and threonine phosphorylation sites in mediating the oxidative stress response. It was expected that sensibility to oxidative stress-induced apoptosis would be enhanced upon introduction of p66<sup>Shc</sup> in non-p66<sup>Shc</sup> expressing cells, similar to what has been reported for p66<sup>Shc</sup>-deficient MEFs (Migliaccio et al., 1999). Surprisingly, p66<sup>Shc</sup> expression did not change the viability of these cells upon treatment with various stress-inducing agents. Although the

experimental setup was changed, no conditions were found in which p66<sup>Shc</sup> expression decreased the viability of these cell lines upon stress induction. The functionality of the HA-p66shc constructs have been proven before in other studies (Faisal et al., 2002; Faisal et al., 2004).

The simplest explanation for these contradictory results would be that we did not find the right experimental conditions, or that we chose the wrong cell lines. LLC-PK1 is a non-transformed proximal tubular epithelial cell line which has been used to study apoptosis in response to oxidative stress (Al-Ghamdi et al., 2004; Allen et al., 2003; Rustom et al., 2003). However, LLC-PK1 cells turned out to be highly resistant to any stress-inducing agents. MCF7 cells are breast cancer cells. It is not clear whether the apoptotic response in transformed cells is regulated by p66<sup>Shc</sup>. However, both cell lines express p53, which becomes stabilized upon treatment with UV or H<sub>2</sub>O<sub>2</sub> (data not shown).

These results could also imply that, in epithelial cells, p66<sup>Shc</sup> is not involved in the regulation of cell viability in response to oxidative stress. To date there has been no report demonstrating a p66<sup>Shc</sup>-dependent regulation of apoptosis in epithelial cells. Other factors specific for epithelial cells could take over the role of p66<sup>Shc</sup> in this regard. One possible candidate is the newly identified protein REDD1 which is a downstream target of p63, a p53 family member (Ellisen et al., 2002). REDD1 was identified as a hypoxia-inducible gene involved in the regulation of cellular ROS (Shoshani et al., 2002). Similar to p66<sup>Shc</sup>, overexpression of REDD1 desensitizes cells to apoptotic stimuli, and loss of REDD1 expression results in reduced intracellular ROS levels and enhanced resistance to oxidative

stress-induced apoptosis (Ellisen et al., 2002; Schwarzer et al., 2005). Remarkably, REDD1 is involved in epithelial development and is specifically expressed in tissues derived from the ectoderm (Ellisen et al., 2002). Therefore, it is possible that REDD1 represents an epithelial-specific factor involved in the regulation of ROS and cell viability. However, to exclude a role for p66<sup>Shc</sup> in this process, more epithelial cell lines should be examined.

### 3.4 Isoform-specific role of p46<sup>Shc</sup>

Beside the isoform-specific role of p66<sup>Shc</sup> in regulating apoptosis, it remains unclear whether p46<sup>Shc</sup> and p52<sup>Shc</sup> display different functions. Both seem to be equally involved in mediating MAPK activation, as shown here and in other reports (Faisal et al., 2004; Ravichandran, 2001). On the other hand, there are some indications for variation in their function. p52<sup>Shc</sup> can be phosphorylated on serine 29 and it has been shown that this phosphorylation is necessary for its binding to the phosphatase PTB-PEST in order to downregulate insulin-induced Erk activation (Faisal et al., 2002). An investigation of the subcellular localization of Shc isoforms revealed a specific and selective localization of p46<sup>Shc</sup> to the mitochondrial matrix (Ventura et al., 2004). Deletion mapping experiments have demonstrated that targeting of p46<sup>Shc</sup> to mitochondria is mediated by its first 32 amino acids, which behave as a *bona fide* mitochondrial targeting sequence. Further, it has been shown that the N-terminal location of the signal peptide is critical for its function. This accounts for the observation that p52<sup>Shc</sup> and p66<sup>Shc</sup>, containing the same sequence but

more internally located, display a remarkably different subcellular localization. These findings indicate that p46<sup>Shc</sup> may exert a non-redundant biological function in signal transduction pathways involving mitochondria.

Another group has reported that p46<sup>Shc</sup> is specifically and heavily phosphorylated in proliferating hepatocytes and cancer cells derived from liver, and localizes in both the nuclei and the cytoplasm of these cells (Yoshida et al., 2004; Yuji et al., 2004). The authors suggest that p46<sup>Shc</sup> localization in the nuclei may be closely related to hepatocarcinogenesis and represents a useful marker for the detection of hepatocytes with high proliferative activity. The same laboratory reported p46<sup>Shc</sup> nuclear localization in gastric normal mucosa and cancer, also suggesting a role for p46<sup>Shc</sup> in gastric carcinogenesis (Yukimasa et al., 2005). However, the question of what is the function of p46<sup>Shc</sup> in the nucleus remains unanswered.

In contrast, Murayama *et al.* (Murayama et al., 2004) demonstrated that antibody ligation of CD9 induced apoptosis of gastrointestinal cancer cell lines in a p46<sup>Shc</sup>-dependent manner.

Taken together, these results indicate that in some tissues p46<sup>Shc</sup> seems to play a more important role than p52<sup>Shc</sup>. Specific conditional knockout studies would help to further our understanding of the physiological role of Shc isoforms.

### 3.5 Conclusion

This thesis introduces a tool for the analysis of single Shc isoforms in tissue culture. Using this tool, we found that p46/p52<sup>Shc</sup>, but not p66<sup>Shc</sup>, play an essential role in a previously unknown signaling pathway downstream of E-

cadherin. This pathway directly links disruption of E-cadherin-dependent cell-cell adhesion to expression of the uPA gene and might therefore play a role in tumor progression. Shc proteins play an important role in mediating MAPK activation. However, receptor tyrosine kinases have redundant ways of coupling to

the MAPK pathway and the individual contribution of various adaptor proteins is not known. Data provided in this thesis suggest that p46/p52<sup>Shc</sup> are exchangeable for growth factor-induced MAPK activation but play a more important role in growth factor-independent MAPK activation.



## 4. MATERIAL AND METHODS

The following chapter adds additional material and methods used in chapter 2 which have not been described before.

**Cell lines.** The renal proximal tubular cell line LLC-PK1 was cultured in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% (v/v) fetal calf serum (AMIMED, Allschwil, Switzerland), 0.2 mg/ml streptomycin, and 50 units/ml penicillin. For serum starvation, cells were incubated in Dulbecco's modified Eagle's medium containing 0.1% fetal calf serum. The human prostate epithelial cell line PNT2, and the human prostate adenocarcinoma cell line PC3, were cultured in RPMI medium supplemented with 10% (v/v) fetal calf serum, 0.2 mg/ml streptomycin, and 50 units/ml penicillin. All cells were grown at 37°C in a humidified incubator with 5% CO<sub>2</sub>. For serum starvation, cells were incubated in their medium containing 0.1% fetal calf serum.

**EGTA treatment.** LLC-PK1 cells were starved for 16 h and then treated with 4 mM EGTA and 1 mM MgCl<sub>2</sub> for an additional 30 min if not indicated differently.

**MTT proliferation assay.** HeLa, PNT2, PC3, and LLC-PK1 cells were transfected with siRNA. The next day, 100-500 cells/well (depending on the cell line) were seeded in triplicate in 96-well plates. The cells were grown in 100 µl medium for 3 days, at which

time 10 µl of a 5 mg/ml stock solution MTT (3-(4,-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium) (Boehringer Mannheim) was added to each well and incubated for an additional 4 h. The MTT is converted to a color crystal product by mitochondrial enzymes, which are then dissolved by adding 100 µl of a 0.01 M HCl solution containing 10% SDS for 8 h. Color development was measured spectrophotometrically on an ELISA reader at a wavelength of 590 nm. Proliferation of control cells was set 100%.

**p46/p52Shc siRNA.** The following 21-mer oligoribonucleotide pair was used as si-p46/p52Shc: 5'-GUG CGG AGA CUC CAU GAG GCC-3' and 5'-CCU CAU GGA GUC UCC GCA CGC-3'. The specificities of these sequences were confirmed by blasting against the GenBank/EMBL database.

**Viability assay.** 1.5x10<sup>6</sup> cells were seeded in 6 cm plates. The next day, first samples were collected (day 0) and the others were starved or treated with EGF, H<sub>2</sub>O<sub>2</sub>, or UV according to the experiment. At the indicated timepoint, cells were trypsinized and diluted in 1 ml of medium, and cell number as well as viability was measured in the Vi-CELL analyzer using the trypan blue exclusion. All samples were done in duplets.

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## 7. ABBREVIATIONS

Ang II	Angiotensin II	MMP	matrix-metallo protease
APC	adenomatous poliposis coli	NGF	neuronal growth factor
CH	collagen homology domain	PBL	peripheral blood lymphocytes
CSR	cytoskeletal reorganization	PDGF	platelet-derived growth factor
DCR	dicer-like protein		
DN	double negative stage	PH	pleckstrin homology domain
DP	double positive stage	PI3K	phosphatidylinositol-3 kinase
dsRNA	double-stranded RNA	PI(4)P	phosphoinositol 4-phosphate
EGF	epithelial growth factor	PI(4,5)P2	phosphoinositol 4,5-diphosphate
EGFR	EGF receptor	PI(3,4,5)P3	phosphoinositol 3,4,,5-phosphate
EMT	epithelia-to-mesenchymal transition	PKB	protein kinase B (Akt)
Erk	extracellular activated kinase	PP2A	protein phosphatase type 2A
FAK	focal adhesion kinase	PTB	phosphotyrosine binding domain
FKHRL	Forkhead family transcription factor	R2D2	dsRNA binding protein
		RISC	RNA-induced silencing complex
Gab	Grb2-associated binding protein	RNAi	RNA interference
GAP	GTPase activating protein	ROS	reactive oxygen species
GPCR	G protein-coupled receptors	Shc	Src-homology and collagen-like protein
Grb2	growth-factor-receptor binding protein-2	Shp2	Src homology phosphatase-1
GTP	guanonsine triphosphate	SHIP	SH2-containing inositol polyphosphate 5-phosphatase
HGF	hepatocyte growth factor		
ICAP-1	integrin cytoplasmic domain-associated protein-1	SOS	son of sevenless
IL	interleukin	SP	single positive stage
IRS	insulin receptor substrate	siRNA	small interfering RNA
IGFR	insulin-like growth factor receptor	SH	src-homology domain
		TCR	T-cell receptor
JNK	jun N-terminal kinase	TCF	T-cell factor
LEF	lymphocyte-enhancer factor	TGF- $\beta$	transforming growth factor- $\beta$
MAPK	mitogen activated protein kinase	uPA	urokinase plasminogen activator
		UV	ultraviolet light
MEFs	mouse embryonic fibroblasts	TPA	12-O-tetradecanoylphorbol-13 acetate
Memo	mediator of ErbB2-driven cell motility		
miRNA	microRNA	VEGF	vascular endothelial growth factor
miRNP	micro ribonucleoprotein particle		

## 8. CURRICULUM VITAE

Sandra Kleiner

### PERSONAL DETAILS

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<b>08.1995-06.2001</b>	Friedrich Schiller University, Jena, Germany	<b>German Diploma (best mark: 1)</b> , Diploma thesis: Development of a model system for the investigation of the c-Met signaling pathway in murine fibroblasts
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**European Life Science Organization Meeting, Nice, France**, 07.2002

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**Event organization**

**Member of the organizing committee of the Career Guidance Conference in Life science** under the hospices of Novartis, Basel, Switzerland, 05.2005

**Co-organization of the traditional Biology Ball**, an event organized by biology students of the 4<sup>th</sup> semester, Jena, Germany, 05.1998

**PUBLICATIONS**

**Kleiner, S.**, Faisal, A., and Nagamine, Y. 2005, J Biol Chem, Induction of uPA gene expression upon blockage of E-cadherin via Src- and Shc-dependent Erk. (*submitted*)

Cramer, A., **Kleiner, S.**, Westermann, M., Meissner, A., Lange, A., and Friedrich KH. 2005, J Cell Biochem, Activation of the c-Met receptor complex in fibroblasts drives invasive cell behavior by signaling through transcription factor STAT3.

Faisal, A., **Kleiner, S.**, and Nagamine, Y. 2004, J Biol Chem, Non-redundant role of Shc in Erk activation by cytoskeletal reorganization.

Kisielow, M\*, **Kleiner, S\***, Nagasawa, M., Faisal, A., and Nagamine, Y. 2002, Biochem J., Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. \*both authors contributed equally

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**PATENT**

Kisielow, M., Kleiner, S., Nagamine, Y. Methods of obtaining isoform-specific expression in mammalian cells. 1-32330A/FMI, 01.2002, Pending EP, JP and US.

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**INTERESTS AND ACTIVITIES**

Outside of laboratory and office work I like physical exercise which includes regularly dancing in our newly built up dancing group "Blickfang" but also outdoor sports like biking, jogging and inline skating. I am interested in foreign languages (Italian, French) and I enjoy learning and speaking them.